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XV. A STUDY OF SOME BIOCHEMICAL COLOUR TESTS. No. I. THE THIOPHENE TEST FOR LACTIC ACID. A COLOUR TEST FOR ALDEHYDES.

By WILLIAM ROBERT FEARON.

(*From the Biochemical Laboratory, Cambridge.*)

(*Received March 12th, 1918.*)

A CHARACTERISTIC of biochemistry is its great heritage of colour tests which have been devised to meet the demand for means of identifying substances of complex or unknown constitution where the more exact methods of pure organic chemistry were of no avail.

Some of these biochemical tests are of a degree of delicacy and specificity almost comparable to biological reactions; but, owing to the difficulties of micro-analysis, the mechanisms of many of these tests are as yet imperfectly understood.

This is not a practical disadvantage as long as the specific nature of the test is assured, but occasionally doubtful cases arise when the knowledge of the rationale of a reaction is essential, and it was from experience of such cases that I was led to investigate some of the well-known colour tests, laying stress on the nature and limiting conditions of the test apart from the chemistry of the substances involved in it.

Hydrocnic Colour Reactions.

Many colour tests are only obtained in the presence of an excess of strong acid, generally sulphuric. In some cases, addition of water after the colour has appeared merely dilutes the tint, but there are certain colour reactions the products of which lose their colour at once if a few drops of water be added.

I have ventured to suggest the term *Hydrocnic*, derived from "Hudōr" (water) and "Okneō" (I shrink), to describe these tests in which the colour is unstable towards water.

These reactions have several points in common, even when they are due to very dissimilar compounds; such as in the Hopkins lactic acid test, Salkowski's cholesterol test, and many of the aldehyde reactions for tryptophan.

A study of *hydrocnic* phenomena calls attention to various important factors capable of modifying or inhibiting colour reactions, as, for example, the local heating due to the addition of the concentrated acid, and the significance of certain impurities in the acids employed.

The Thiophene Test for Lactic Acid.

This test has been selected as it is a typical *hydrocnic* reaction and, though it involves no complex chemical substances, its mechanism has not yet been fully explained.

The test was introduced by Hopkins and was first described in Cole's *Physiological Chemistry* in 1904.

The test is very sensitive to the presence of water in two ways. If the sulphuric acid used be at all dilute or a little water be added with the lactic acid, the solution on warming becomes yellow and does not give the cherry-red colour on the addition of the thiophene. Again, if a few drops of water be added after the red colour has developed it is at once discharged.

In his description of the test, Hopkins [Hopkins and Fletcher, 1907] observes that it is also given by acetaldehyde and by glyoxylic acid.

It is well known that lactic acid when heated with sulphuric acid is decomposed into acetaldehyde and formic acid, or, if an oxidising agent be present, into pyruvic acid and water.

(1) $CH_3 . CH(OH) . COOH \longrightarrow CH_3CHO + HCOOH.$

(2) $CH_3 . CH(OH) . COOH + O \longrightarrow CH_3 . CO . COOH + H_2O.$

G. Denigès [1909] has employed alcoholic guaiacol to show this formation of aldehyde, and Ryffel [1909] has used it for the estimation of lactic acid in urine.

The results of the action of sulphuric acid in varying proportions on lactic acid were investigated.

Expt I. 20 cc. lactic acid and 20 cc. pure concentrated sulphuric acid, with a crystal of copper sulphate, were mixed. The liquid gradually darkened and gas was evolved. When the reaction had subsided the mixture was distilled under reduced pressure, from a water-bath at 80°.

The distillate was collected in 20 cc. water. It consisted of formaldehyde, acetaldehyde chiefly, formic acid, acetic acid. The gas evolved was CO_2 with some CO.

Expt II. 10 cc. lactic acid and 20 cc. sulphuric acid as before. This time there was a considerable amount of acetaldehyde in the distillate. Not much gas was evolved.

Expt III. 20 cc. moist lactic acid (containing about 10 % water) and 10 cc. sulphuric acid. The distillate contained only a trace of aldehyde but gave a fairly distinct red coloration with ferric chloride.

There was a great evolution of gas (chiefly CO) during the reaction.

The thiophene lactic acid test was then performed on a drop of each of the distillates. I and II gave the cherry-red, III gave a faint orange.

Each of the substances identified in the distillate was then tested separately, when it was found that both formaldehyde and acetaldehyde gave a colour with thiophene in the presence of excess of sulphuric acid.

With regard to the poor production of aldehyde in the last experiment, it was found that acetaldehyde is much less stable in moderately dilute sulphuric acid than in strong acid, consequently it is necessary in performing the lactic acid test to see that there is no appreciable amount of water present at the outset, otherwise the acetaldehyde will be destroyed as it is produced and the thiophene will give no coloration.

It is this decomposition of aldehyde that probably accounts for the 10 % error in Ryffel's method for estimating lactic acid in urine.

A Colour Test for Aldehydes.

Working along the lines of the previous test, the following reaction for aldehydes was devised.

A couple of drops of a 0·2 % alcoholic solution of thiophene are added to 5 cc. concentrated sulphuric acid (which must be free from nitrous and nitric contaminations) and mixed. Then, if a drop of a weak solution of an aldehyde be added, a red colour develops and spreads through the acid. A few drops of water discharge the colour but it returns on the addition of more sulphuric acid.

The test is very delicate with most aldehydes. 1 part CH_2O in 10,000 parts H_2O gives a deep plum-coloured ring; 1 part in 100,000 gives a distinct band.

The colour varies slightly with the different aldehydes. Formaldehyde gives a purple red, acetaldehyde a cherry-red, acrolein a rose carmine. The test is given also with the substituted aldehydes, such as chloral, *p*-hydroxybenzaldehyde, etc. All these colours are typically hydrocnic.

By the aid of this test, the presence of aldehydes in moist ether can be demonstrated. If some aqueous ether be left in the sunlight for a few days it

gives an aldehyde reaction with the sulphuric acid and thiophene. This fact is of considerable importance since ether is frequently used in the extraction of lactic acid. The ether employed should be fresh and dry and the subsequent evaporation should not be done on a water-bath but at ordinary temperature. It is well known that moist ether, when warmed, readily oxidises with production of acetaldehyde and other substances.

The Interaction of Thiophene and Aldehydes.

Amongst the early work on thiophene two condensations with chloral and bromal, in the presence of glacial acetic acid and sulphuric acid, are recorded by V. Meyer [1883] and A. Peter [1884]. The formula ascribed is

$$CCl_3 . CH : (C_4H_3S)_2.$$

for the chloral derivative.

Töhl and Nahke subsequently [1896] described a condensation between thiophene and benzaldehyde with the formation of dithienylphenylmethane;

$$C_6H_5 . CH : (C_4H_3S)_2.$$

On mixing thiophene and acetaldehyde in light petroleum in the molecular proportions corresponding to a body, $CH_3 . CH : (C_4H_3S)_2$, and adding phosphorus pentoxide, the pentoxide turned deep purple and a reaction began. The products were extracted with alcohol, ether, and light petroleum, respectively. The extracts were neutralised, washed, dried and fractionated, or fractionated straight away.

From two of the extracts a liquid fraction was obtained which gave a deep crimson with sulphuric acid.

The fraction was an orange liquid which boiled with decomposition at 121°, was insoluble in water, and had a peculiar smell.

The determination of the molecular weight and the composition of this substance was deferred until an adequate supply of thiophene could be obtained.

SUMMARY.

1. It is proposed to introduce the term *hydrocnic* for biochemical colour reactions, the products of which are decolorised by the addition of small quantities of water.

2. The thiophene reaction for lactic acid is due to the production of formaldehyde and acetaldehyde from the lactic acid, which interact with the thiophene in the presence of excess of sulphuric acid to give the cherry-red colour.

3. A new colour test for aldehydes is described depending on the fact that they interact with thiophene in the presence of sulphuric acid to give coloured substances.

4. A preliminary reference is made to the reaction between aldehydes and thiophene.

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XVI. THE ROLE OF THE LIVER IN HIPPURIC ACID SYNTHESIS.

By E. LACKNER, A. LEVINSON and WITHROW MORSE.

From the Nelson Morris Memorial Institute for Medical Research, Michael Reese Hospital, Chicago.

(*Received March 19th, 1918.*)

The attempt will be made in the following pages to show that by the use of hydrazine, a specific agent affecting liver tissue and leaving that of the kidney intact [Wells, 1908; Underhill and Kleiner, 1908], it is possible to demonstrate a considerable participation of the liver in hippuric acid conjugation. The studies of Bunge and Schmiedeberg [1876], followed by the corroborating studies of Kochs [1879] and of Bashford and Cramer [1902], have been interpreted as decisive evidence of a difference in power of effecting hippuric acid pairing on the part of carnivores, including man, and herbivores, in that the seat of the process in herbivores is not limited to the kidney as in the case of the carnivores. This conclusion remained practically unchallenged until Kingsbury and Bell [1915, p. 301] found "a relatively large amount of hippuric acid in the liver of a nephrectomised dog after injection of glycocoll and sodium benzoate" suggesting "that the liver like that of the rabbit may possess this synthetic power."

In the present set of experiments we have departed from the procedure followed by other investigators in two respects, namely, that we have utilised the urine rather than the blood, and secondly, we have employed a reagent which may be used subcutaneously to cause liver involvement while leaving the kidney intact without introducing the additional factor of operation, ligaturing, etc.

Methods.

Two male dogs, weighing respectively 9·05 and 9·50 kg., were isolated in metabolism cages. The urine was collected in 24 hour quantities, thymol in alcohol being used as a preservative; faeces were excluded by use of glass-wool and filter plates. The food consisted of dog-biscuits supplemented by fresh beef, chicken trimmings and boiled meat from the soup kitchen. The intake of water and of chloride was noted; likewise we noted carefully the general condition of the dogs especially after the administration of reagents. The dogs

were kept in the cages several days before the collection of urine was commenced in order that they might become accustomed to their surroundings and also that their controlled food might replace that previously given them.

In the administration of sodium benzoate (2 g.) and of hydrazine sulphate (500 mg.) by mouth, we wrapped the substances in fresh steak formed into a bolus which the dogs swallowed freely, thus insuring that the animals received the complete amount of the drug. Subcutaneous injections of 500 mg. hydrazine sulphate dissolved in 20 cc. of sterile water were given into the flanks under aseptic conditions.

Hippuric acid was determined in 50 cc. aliquots of urine after the method of Folin and Flanders [1912], the reduction in amount of urine from 100 cc. to 50 cc. being compensated for by reducing the amounts of the other reagents, nitric acid, ammonium sulphate, sodium chloride, chloroform, etc., correspondingly.

Chlorides were determined by the Volhard method, for renal efficiency. Amino-nitrogen was determined gasometrically upon the hydrolysed urine. Free benzoic acid was determined upon the residue from 50 cc. of evaporated urine; the dry residue was transferred to an allundum thimble and extracted in a Soxhlet apparatus for four hours with chloroform as solvent. Titration was then performed with decinormal sodium ethylate from sodium, as in the case of the hydrolysed hippuric acid determinations. On the thirty-fourth day the dogs were killed by means of coal gas and autopsied.

Results.

The following table gives the results for the two dogs extending over a period of 34 days; the quantities are calculated for 24-hour periods:

Day	Dog	Hippuric N.	Amino N.	NaCl	24 hour quantity urine	
1	1	0·014 mg.	0·035 g.	1·45 g.	150 cc.	
	2	0·023	0·749	0·96	130	
2	1	0·026	4·190	3·07	360	
	2	0·049	2·120	0·28	300	
3	1	None	None	None	None	
	2	0·029	„	0·58	100	
4	1	0·014	0·190	5·90	100	
	2	0·040	0·888	0·25	550	
5	1	0·010	0·190	1·40	150	
	2	0·026	0·526	0·19	275	
6	1	None	None	None	None	
	2	0·119	2·15	0·39	500	
7	1	0·083	3·51	2·80	500	
	2	0·037	0·800	0·25	500	
8	1	None	None	None	None	2 g. sodium benzoate
	2	„	„	0·12	400	

Day	Dog	Hippuric N.	Amino N.	NaCl	24 hour quantity urine	
9	1	0·174	3·05	3·20	425	
	2	0·091	None	0·51	225	
10	1	0·030	1·05	1·70	250	
	2	0·076	0·945	0·28	450	
11	1	0·054	1·91	2·10	230	
	2	0·040	0·314	0·27	260	
12	1	0·058	2·22	3·50	400	
	2	0·074	1·860	None	380	500 mg. hydrazine sulphate per os.
13	1	None	None	,,	None	
	2	,,	,,	0·17	375	
14	1	.	1·70	1·40	250	
	2	0·089	1·97	0·26	400	
15	1	0·010	0·124	1·20	140	
	2	0·132	0·895	0·26	500	
16	1	0·100	1·43	3·50	450	
	2	0·29	1·87	0·14	260	
17	1	None	None	None	None	
	2	0·017	0·776	0·16	250	
18	1	0·071	None	3·20	450	
	2	0·026	,,	0·15	280	
19	1	None	0·22	3·60	500	
	2	0·020	0·717	0·22	400	500 mg. hydrazine sulphate per os.
20	1	0·010	1·00	None	150	
	2	0·013	1·134	,,	160	
21	1	None	None	0·50	200	
	2	,,	,,	0·12	180	
22	1	0·018	,,	0·51	150	
	2	0·028	1·05	0·41	150	
23	1	0·024	1·37	None	180	
	2	0·024	None	,,	350	
24	1	None	,,	0·82	130	
	2	,,	,,	0·18	150	
25	1	0·036	,,	0·37	450	
	2	None	,,	0·21	250	500 mg. hydrazine sulphate sub-cutan.
26	1	0·016	0·504	0·92	250	
	2	0·018	1·091	0·37	150	2 g. sodium benzoate
27	1	0·099	1·74	0·45	250	
	2	0·051	1·728	None	250	
28	1	0·020	1·740	0·26	200	
	2	None	None	0·13	225	
29	1	0·028	2·33	None	375	
	2	0·015	0·981	,,	130	
30	1	0·014	0·733	,,	100	
	2	0·042	2·52	,,	325	
31	1	0·010	None	0·83	100	
	2	0·026	2·106	None	300	
32	1	None	None	1·73	350	
	2	,,	,,	0·072	225	500 mg. hydrazine sulphate sub-cutan.
33	1	0·013	0·075	0·75	160	
	2	None	0·114	0·10	200	
34	1	0·017	None	0·43	300	
	2	0·014	0·353	0·07	250	2 g. sodium benzoate

Professor O. T. Schultz, who kindly performed the autopsies and examined the slides from formalin and Zenker fixations of portions of the livers and kidneys of the two dogs, reports that both dogs showed marked fatty degeneration of the liver, while the kidneys were intact.

Discussion.

We may summarise the results by averages of the amounts of the various substances excreted during the different periods of the experiments.

Average of excretions previous to administration of sodium benzoate or hydrazine:

Dog	Hippuric N.	Amino N.	Chlorides	Quantities
1	0·031	1·98	2·92	252
2	0·043	1·17	0·45	332

Average after giving 2 g. of sodium benzoate:

1	0·085	2·00	2·33	301
2	0·069	0·629	0·29	301

Average after giving hydrazine sulphate by mouth:

1	0·059	1·36	2·48	323
2	0·104	1·47	0·019	349

Average after a second feeding of hydrazine sulphate:

1	0·017	0·863	1·35	218
2	0·021	0·969	0·23	231

Average after injection of hydrazine subcutaneously:

1	0·026	0·50	0·64	350
2	0·018	1·091	0·29	200

Average after feeding sodium benzoate following hydrazine injection:

1	0·040	6·54	0·51	203
2	0·033	1·83	0·13	246

Average after a second injection of hydrazine:

1	0·013	0·075	1·24	255
2	None	0·57	0·086	213

Average after a final feeding of benzoate following hydrazine injection:

1	0·017	None	0·43	300
2	0·014	0·353	0·07	250

From these results, we see that following the feeding of sodium benzoate initially after establishing the normal excretion, the hippuric acid nitrogen is increased. This is true after we subtract the free benzoic acid figure from the total amount determined after hydrolysis, namely, 0·026, leaving 0·065 mg in the case of Dog 1. After the feeding of hydrazine by mouth, upon the first

occasion, there is no apparent disturbance of hippuric acid excretion, such as we find in the case of the second feeding. There is an apparent reduction of the amount of hippuric acid after the injection of hydrazine subcutaneously, but inasmuch as we find a rise in excretion following the administration of sodium benzoate, we may conclude that no extensive damage has been done to the liver up to this point. After a second injection of hydrazine subcutaneously, there is a marked fall in the case of Dog 1, the figures for the second dog being lost due to defect in the water system passing through the reflux condensers during hydrolysis. Finally, after the feeding of sodium benzoate after the second injection of hydrazine, a marked fall in hippuric acid excretion is observed. Obviously, we are not able to say how great a difference in liver involvement has occurred between the twenty-fifth day and the final day of the experiment, but from the figures, it is evident that the damage has been progressive.

During these periods, the kidneys have remained intact, as we may observe from the chloride excretion; in studying these figures, it must be borne in mind that during the last periods of the experiments, the dogs took less and less food, so that the chlorides appeared in progressively smaller amounts. However, a comparison of the figure for the chlorides excreted by Dog 1 during the first period and during the next from the last, namely, a comparison of 2·92 g. with 1·24 g., makes it evident that after it has become established that the liver is damaged, the kidneys are excreting quantities of chlorides not far from what we should expect, taking into consideration the lessened intake in the food. The chemical figures are fully borne out by the study of the histological sections.

We were disappointed in finding that the amino-nitrogen could not be correlated with the hippuric acid figures. At best, the glycocoll fraction is not large and might easily be overbalanced by changes in the amino-nitrogen fraction due to the degenerative changes of the liver. Henriques and Sörensen [1909] have utilised the glycocoll fraction after hydrolysis of hippuric acid, the determination being made by means of the formaldehyde-titration method.

From the figures representing the 24 hour quantities of urine, it is apparent that there is no effect either as diuretic or anuretic exerted by the reagents used in the experiments. Consequently, the results are not vitiated by these factors, which Astolfoni [1905] found operative in his experiments, hippuric acid being excreted in increased amounts following the administration of diuretics and vice versa.

The figures obtained upon free benzoic acid in the urine show that there is

no effect upon the relative amounts of hippuric acid estimated as benzoic acid after hydrolysis, by this factor, so that the criticism of Lewiniski [1908] cannot be applied to this work.

The conclusion that the liver plays an important role in hippuric acid synthesis is well in keeping with what we know of the functions of the liver, as contrasted with those of the kidney. There are a number of conjugations performed in the liver. Thus, various syntheses are known involving the acids, such as phosphoric acid, sulphuric acid, glycuronic acid, etc. Conjugations with sulphuric acid lead to the formation of the so-called ethereal sulphates; pairing of glycuronic acid with substances of various sorts leads to the formation of glucoside compounds. Conjugation of glycocoll occurs with any compound which after oxidation leads to the formation of benzoic acid (salicylic acid, *p*-hydroxybenzoic acid, etc.), the end-product being the so-called "ur-acids" of acid-amino nature. In birds, the formation of dibenzoylornithine, where ornithine takes the place of glycocoll in hippuric acid syntheses, is known to take place principally in the liver. In the dog, a subsequent pairing of hippuric acid and urea takes place in the liver. This does not necessarily show that the primary conjugation to form hippuric acid occurs in the same organ as the secondary pairing, yet there is reason to believe that this is the case. The alleged hippuric acid synthesis is the exception to the rule concerning one of the characteristic functions of the liver, if we adopt the conclusions of Bunge and Schmiedeberg and their school as final.

Conclusions.

Data are presented to show that the liver participates in hippuric acid conjugation to a marked extent. The data have been derived by poisoning the liver with hydrazine, leaving the kidneys intact. The conclusions reached in this investigation are borne out by the work of Kingsbury and Bell and are aligned with our knowledge of the characteristic synthetic functions of the liver as contrasted with those of the kidney.

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XVII. ON THE CONCENTRATION OF ANTITOXIC SERA BY THE SALTING OUT OF THE HEAT-DENATURATED SERUM PROTEINS WITH SODIUM CHLORIDE.

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A RECENT investigation of the factors influencing the concentration of antitoxic sera by methods involving the use of ammonium sulphate has shown that the degree of concentration attained is regulated by the extent of the denaturation of the serum proteins induced during the heating of the serum. There is, however, a limit to the degree of concentration. In cases where the heat-denaturation is greater than about 50 %, the attempted separation of the antitoxin from the heated sera by means of ammonium sulphate leads to the production of end products showing a considerable loss of antitoxin. The missing antitoxin is carried down with the First Fraction precipitates (i.e. at 30 % of saturation with ammonium sulphate) and for its recovery it is necessary to extract those precipitates with brine [Homer, 1917, 1].

Even in our routine work where the heat-denaturation of the serum proteins is of the order of 30 %, we have found it advisable, for reasons based on the above observations, to resort to the extraction of the First Fraction precipitates with brine. As the adoption of this procedure necessitates the use of two "salting out" agents it seemed worth while to ascertain whether the process of concentration could be shortened by treating the heated sera directly with sodium chloride, thus dispensing with the use of ammonium sulphate.

In this connexion it was necessary to make a further study, not only of the factors influencing the heat-denaturation of the serum proteins, but also of the extent to which the denaturation can be carried without rendering the protein-antitoxin complex insoluble in brine. The work, which is still in progress, has

shown that the extent of the heat-denaturation of the serum proteins depends on:

(1) The reaction of the serum or plasma previous to its being heated [Homer, 1917, 2].

(2) The temperature at which the heating is conducted.

In all cases the maximum values are more quickly reached in acid than in alkaline sera and, irrespective of the [H·] of the sera, the time taken is far less at high than at low temperatures.

It was also found that the maximum values for the heat-denaturation at a specific reaction and at a specific temperature could be increased by the addition of substances such as phenol and its homologues, ether, chloroform, or electrolytes to the serum previous to its being heated.

The knowledge thus gained has been applied to the present investigation. Pending the completion of a series of experiments dealing with the effect of heating sera at temperatures ranging from 58 to 72° on the extent of the heat-denaturation of the serum proteins, and the bearing of the latter on the solubility of the thus denaturated proteins and on the inactivation of antitoxin, my experiments have been conducted at temperatures between 57° and 58°, for, it has been shown that during the prolonged heating of sera at this temperature there is no actual destruction of antitoxin even in those cases in which an extensive heat-denaturation of the serum proteins has been induced.

In order to ensure the desired heat-denaturation, the plasma, previous to being heated at 57–58°, was subjected to one of the following methods of treatment:

(I) The reaction was suitably adjusted by the addition of acid or alkali;

(II) To the plasma was added 2 % of sodium chloride followed by fixed amounts of phenol or its homologues; or

(III) To the plasma were added various amounts of electrolytes.

The treated plasma was then heated at 57–58° for the necessary time. At the end of the heating period (*vide infra*) there were added to the hot serum twice its volume of a saturated solution of sodium chloride and a sufficient amount of solid salt to ensure complete saturation of the brine-serum mixture[1]. The liquids were allowed to stand at room temperature for several days.

It was found that after the mixtures had stood for two to three days there was then, practically speaking, a complete extraction of the antitoxin

[1] Tested by means of a hydrometer.

from those sera which, during the heating, had remained clear or had become opalescent, or of which the consistency had been changed into that of a thin suspension unfilterable through paper. In those cases in which the consistency of the serum had been changed to that of a thick suspension, due to the actual precipitation of particles of heat-denaturated protein, a much longer time was required for the extraction of the antitoxin by the brine. Thus, with the sera showing a heat-denaturation of 40 to 45 %, about 6 to 8 days' extraction was sufficient for the extraction of the antitoxin. On the other hand, even after several weeks' extraction, the recovery of antitoxin was far from complete from those sera in which a more extensive heat-denaturation and correspondingly increased precipitation of heat-denaturated protein had been induced.

After the brined liquids had stood at room temperature for the necessary length of time they were filtered. Except where phenol and cresylic acid had been added, there was slow filtration of the brined extract of those sera of which the reaction, previous to the heating, lay between P_H 5·5 and 7·2. The slow filtration was due to the presence of heat-denaturated protein in a semi-gelatinous condition. The difficulty in filtration was obviated by gradually warming the liquids to a temperature of 45°, thereby inducing the coagulation of the particles of protein into aggregates of sufficient dimensions to enable their separation to be effected by filtration through paper.

The precipitates were washed with brine and the washings, after filtration, were added to the main bulk of the filtrates to which was then added 0·25 % of its volume of glacial acetic acid. The ensuing precipitate was filtered, drained, pressed and after the addition of 3 % of washing soda was dialysed until the salt content was the same as that of the water in the dialysing tank. To the residue from dialysis was added 1 % of sodium chloride and 0·35 % of cresylic acid.

The end products were clear and readily filterable, for, in contrast to those obtained by methods involving the use of ammonium sulphate they were not liable to contain particles of protein in colloidal suspension.

On the other hand they showed a lower percentage removal of the serum proteins and a consequent lower degree of concentration than is now obtained in the Banzhaf [1913] and the Homer [1916] methods.

The less complete removal of the serum proteins can be attributed mainly to the fact that the addition of 0·25 % of glacial acetic acid to the brined sera leads to the precipitation of a considerable amount of albumin with the pseudoglobulin. As the antitoxin is attached to the latter, the presence of the former must reduce the degree of concentration to a value considerably less

than would have been obtained had the precipitation of albumin at this stage been avoided.

The observations that were made during the progress of the work have presented further points of interest with regard to the properties of heat-denaturated serum proteins. They are dealt with in detail below and may be of assistance to those engaged in problems connected with the concentration of sera.

I. The salting out of sera in which the heat-denaturation has been regulated by an adjustment of the reaction.

The hydrogen ion concentration [H·] was adjusted to values ranging from P_H 8·7 to 4·2 by the addition of the necessary amounts of ammonia, acetic or sulphuric acids respectively, the determinations being made in a Walpole's colorimeter with the indicators suitable for the purpose [Homer, 1917, 4].

The alkaline sera were heated to a temperature of 57–58° for a period of not less than four hours; the acid sera were kept at this temperature for two hours only.

At the conclusion of the heating the amounts of acid or of alkali initially added to the sera were respectively neutralised. The liquids were then treated with solid sodium chloride and with brine in the manner described above.

It was found that the success of the operation was influenced by the nature of the changes induced during the heating of the serum. As the extent of the change was primarily regulated by the [H·], comparisons were made of the results from the concentration of those sera of which previous to their being heated the [H·] had been adjusted to values between P_H 8·7 and 5·0, 5·0 and 4·7 and to values more acid than P_H 4·7.

(*a*) *The concentration of sera of which the* [H·] *had been adjusted to values between* P_H 8·7 *and* 5·0.

In Table I have been embodied the results obtained from the concentration of such sera.

It will be seen that, where the [H·] had been adjusted by the addition of acetic acid or of ammonia, there was a sufficient recovery of antitoxin in the final products to warrant the application of the method to the routine concentration of sera. As was to be expected a further recovery of antitoxin could be made by a second extraction of the precipitates with brine.

However, in those cases in which sulphuric acid had been employed to adjust the [H·] to values between P_H 5·2 and 5·0, the recovery of antitoxin in

the end product was considerably less than where the reaction had been adjusted approximately to the same values by the addition of acetic acid. It was found that, although, in the two sera, the extent of the heat-denaturation was approximately the same, the percentage removal of the serum proteins was considerably more from those sera acidified with sulphuric acid than from those to which acetic acid had been added.

TABLE I.

Showing the results obtained from the concentration of antitoxic sera by the direct treatment of the heated sera with salt. The extent of the heat-denaturation had been regulated by an adjustment of the $[H^{\cdot}]$ *to values between* P_H 8·7 *and* 5·0.

Reaction of the plasma adjusted by the addition of :—	P_H of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original serum removed during the process of concentration	Percentage of the total antitoxic units of the original serum recovered in the residue from the dialysis of the proteins precipitated by the addition of acetic to the filtered brined sera	Increased potency per c.c. shown by the end products calculated, for the purpose of comparison, on the basis that they contain 20 % of protein
Acetic acid	5·4	16	26	—†	—
,,	5·2	27	35	> 90	4
,,	5·15	30	40	—†	—
,,	5·1	33	43	—†	—
,,	5·08	36	43	86	4·5
,,	5·05	40	58*	75	4
,,	5·00	43	54	88	5
Sulphuric acid	5·18	33	54	65	5
,,	5·05	43	80	< 50	< 3
Ammonia	8·3	35	46	84	5
,,	8·5	40	55	88	5
,,	8·7	42	54	—*	—

* Brined plasma which had stood at room temperature for two days only.

† Not tested owing to the scarcity of experimental animals.

It is thus evident that there are factors other than the $[H^{\cdot}]$ which affect the nature of the changes induced during the heating of sera. It is possible that the above increased precipitation was due to the more marked effect of the divalent "sulphions" than of the monovalent "acetions" on the *electrical* relationship between the heat-denaturated protein and the solution; a relationship which is recognised as a determining factor in the precipitation of a colloid.

(*b*) *The concentration of sera of which the* $[H^{\cdot}]$ *had been adjusted to values between* P_H 5·0 *and* 4·7.

In these cases the consistency of the heated sera was that of a thick suspension due to the precipitation of heat-denaturated protein in a filterable form.

It was ascertained that, during the process of concentration, the recovery of antitoxin in the end products decreased as the acidity of the sera was increased. At the same time there were indications that the recovery in brine was more satisfactory from those sera in which the reaction had been adjusted by means of acetic acid than where sulphuric acid had been used.

Thus, it was found that with sera acidified with acetic acid, and showing a heat-denaturation of about 50 %, the extraction of the heated sera with brine for two days led to the recovery of about 50 % of the antitoxin; after seven days the recovery amounted to 66 %. Where the heat-denaturation had been of the order of 70 %, the treatment of the heated sera with brine for seven days led to the recovery of about 30 % of the antitoxin. In neither case was there a further increase in the percentage recovery of the antitoxin by a prolongation of the time of extraction to a period of five weeks.

On the other hand with sera acidified with sulphuric acid, in which a heat-denaturation of 35 to 40 % had been induced, there was a recovery of only 66 % of the total antitoxin after seven days' extraction with brine. Where the denaturation had been of the order of 65 % the recovery was less than 10 % in the same time. In neither case was the extent of the recovery of the antitoxin appreciably improved by a prolongation of the time of extraction to five weeks.

The more marked insolubility of the heat-denaturated proteins in brine induced during the heating of sera to which sulphuric acid had been added, is shown by the following experiment.

The reaction of antitoxic plasma was adjusted by means of acetic acid and of sulphuric acid respectively so as to ensure a heat-denaturation of about 60 %. The adjusted sera were then heated for two hours at 57–58° by which time they had become semi-solid in consistency. In each case after the neutralisation of the acid there was added an equal volume of a saturated solution of sodium chloride, and the mixture was well shaken and filtered at once. The precipitates were well drained and, after being freed from adhering fluid by gentle pressure between cloths and boards, were triturated in a mortar with 50 cc. of a saturated solution of salt. The thin paste was then diluted with the salt solution to a volume six times that of the original plasma. The liquids were kept at room temperature in stoppered flasks and were well shaken from time to time.

After standing for stated periods of time, samples of the liquids were filtered and the protein and antitoxin contents of the filtrates were measured. The results showed that at the end of one week the brine had redissolved an

appreciable amount of the heat-denaturated protein precipitated from those sera to which acetic acid had been added. Animal tests showed that, to this redissolved protein, was attached about 50 % of the total antitoxin taken for concentration. The amount of protein and antitoxin thus recovered from the precipitate was not appreciably increased after a further twenty-one days' extraction.

On the other hand none of the filtered brine extracts of the protein precipitated from the sera acidified with sulphuric acid contained more than a trace of protein even after four weeks' extraction; the antitoxin recovered was considerably less than 10 % of the original.

(*c*) *The concentration of sera of which the* [H$^{\cdot}$] *was more acid than* P_H 4·6.

To separate volumes of antitoxic sera were added the requisite amounts of acetic and of sulphuric acids respectively to bring the reaction to the value P_H 4·4. The sera were then heated for two hours at 57–58°, and, after the neutralisation of the acid, the semi-solid liquids were treated with salt and with brine in the manner described above. Samples of the brined sera were taken at stated periods and the antitoxic content of the filtrates were compared.

After ten days traces only of the protein had been redissolved; the amount of antitoxin in solution was considerably less than 10 % of the original. There was no improvement in the recovery of antitoxin effected by a prolongation of the time allowed for extraction to four weeks.

The precipitated heat-denaturated proteins from the sera thus acidified, were triturated with brine in the manner described above. Even after several weeks' extraction in a volume of brine six times that of the original serum, there was redissolved practically none of the protein precipitated from the sera acidified with sulphuric acid, and not more than a trace where acetic acid had been used. In both cases the filtered brine extracts contained considerably less than 10 % of the original antitoxin of the serum.

The observations recorded under (*a*), (*b*) and (*c*) thus indicate that there is a greater production of "salt-insoluble" protein during the heating of sera of which the [H$^{\cdot}$] has been adjusted to values more acid than P_H 5·4 by the addition of sulphuric acid than by that of acetic acid. They also demonstrate that, irrespective of the acid used, an increase in the degree of acidity is followed by an increase in the precipitation of heat-denaturated protein and a corresponding decrease in the recovery of antitoxin in a form in which it can be rendered suitable for use clinically.

It is thus evident that, for the successful concentration of sera by the method under discussion, it is advisable to adjust the reaction of the plasma to values about P_H 8·3 or about 5·0. For this purpose a routine method has been suggested [Homer, 1917, 4] in which use is made of the indicators *α-naphtholphthalein* and *dibromo-o-cresolsulphonephthalein*.

Since slight variations in the reaction round about P_H 8·3 are not followed by the marked variations in the heat-denaturation and precipitation of the serum proteins so characteristic of sera more acid than P_H 5·5, preference has been given to the adjustment of the $[H^{\cdot}]$ to the former value.

II. The "salting out" of sera in which the heat-denaturation had been conducted in presence of cresylic acid.

As the use of trikresol[1] has been of such practical value in improving the concentration of sera by the Banzhaf [1913] and the Homer [1916] processes, experiments were undertaken to ascertain whether its use in the method under consideration would be followed by similar satisfactory results.

Experimental concentrations were carried out as follows.

To plasma containing 2 % of solid sodium chloride were added fixed amounts of cresylic acid. The reaction of the plasma was then adjusted to values ranging between P_H 7·2 and 8·5 previous to its being heated. The heated plasma, after being treated with solid salt and with brine in the manner described above, was allowed to stand at room temperature for at least six days to ensure the extraction of the antitoxin.

It was found that the antitoxin could be completely recovered by the brining of sera containing 0·35 % of cresylic acid or less which had been heated to 57–58° for periods of from four to six hours. The addition of cresylic acid in a greater amount led to an increased production of "salt-insoluble" protein; during the process of concentration, the antitoxin attached to this insoluble protein was lost. Thus, the filtered brine extracts of those sera containing

[1] Since the publication of the papers on this subject, we have read in the current literature an abstract of a paper by Banzhaf in which, for the improvement of his technique, he recommends the use of trikresol in an amount somewhat greater than that advocated by us.

We have recently ascertained that the amount of trikresol necessary for the production of clear end products, without inducing undue losses during the process of concentration, depends on the composition of the sample used. Thus with that in use during the years 1916–1918, it was necessary to add 0·30 % to the serum; with a recent consignment of the substance only half this amount was required. It is therefore advisable for those in charge of the concentration of sera to ascertain experimentally, with the trikresol at their disposal, the minimum amount necessary to give the desired results.

In our routine work we have not found it advisable to raise the temperature of the serum mixtures to the high limit now suggested by Banzhaf [1918].

0·5 % of cresylic acid showed a loss of about 15 % of the antitoxin; with 0·75 % of this substance the loss was somewhat greater. The percentage recovery of antitoxin was in no way increased by a prolongation of the time allowed for extraction or by an increase in the volume of brine added to the sera.

Appreciable losses were also experienced in the concentration of sera containing 0·30 to 0·35 % of cresylic acid in which the heating had been conducted at temperatures above 60°.

The losses were, in part, due to the precipitation of antitoxin with the "salt-insoluble" protein and, in part, to an actual inactivation of antitoxin. The extent to which the cresylic acid influences these factors is in the course of investigation and will be dealt with in a future communication.

During the course of the concentration of sera containing cresylic acid it was noticed that, irrespective of the [H·] of the batches of sera, the brined extracts of the heated sera filtered readily. The ease of filtration was probably due to the stimulating effect of the cresylic acid on the aggregation of the heat-denaturated protein into particles of sufficient dimensions to ensure their separation by filtration through paper.

TABLE II.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denaturated in presence of cresylic acid, with salt.

	Percentage of cresylic acid added to the plasma	P_H of the plasma previous to its being heated	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage of the total proteins of the original plasma removed during the process of concentration	Percentage of the total antitoxic units of the original plasma appearing in the residue from the dialysis of the proteins precipitated by acetic acid from the filtered brined sera	Increased potency per c.c. shown by the end products, calculated for purposes of comparison on the basis that these contain 20 % of protein
A	0·00	8·3*	40	46·8	about 85†	5
	0·30	7·4	22·3	51·1	„ 85†	4
	0·30	8·0*	42·0	46·8	„ 85†	4
B	0·00	7·6	18·2	55·4	> 90†	5
	0·25	7·6	36·5	48·0	> 88†	4·6

* Reaction adjusted by the addition of ammonia.

† Not tested out because of the shortage of experimental animals.

The data with regard to concentrations of plasma containing 0·30 % of cresylic acid have been embodied in Table II. They show that, in spite of the increased heat-denaturation induced by the addition of cresylic acid, there was no appreciable increase in the percentage removal of the serum proteins. For this reason the degree of concentration did not show any improvement on that obtained in control experiments with the same sera containing no cresylic acid.

This anomaly was eventually shown to be due to the fact that, while the cresylic acid increases the precipitation and removal of heat-denaturated pseudoglobulin, it also induces an increased precipitation of albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

III. The "salting out" of sera in which the heat-denaturation had been induced in the presence of electrolytes.

It has been demonstrated [Homer, 1916] that there is no appreciable destruction of antitoxin during the prolonged heating of antitoxic sera, to which electrolytes have been added, at 57-58°. As a detailed study has already been made of the effects produced by the addition of definite amounts of sodium chloride to the serum previous to its being heated, attention has been confined in the present investigation to the addition of sulphates to the plasma. For, in view of the more marked tendency of the "sulphion" (*ante*, p. 194) to render the particles of heat-denaturated protein insoluble in brine, it was thought that their addition to the plasma might lead to a greater removal of the serum proteins, and consequent increased degree of concentration, than had been obtained in sections I and II.

Experimental concentrations were accordingly undertaken in which to fixed volumes of the plasma were added definite amounts of the sulphates of ammonium, sodium, potassium, magnesium and zinc respectively. The liquids after being heated at 57–58° for periods of from four to six hours were treated with salt and with brine according to the directions given above; the brined sera were allowed to stand at room temperature for periods of from ten to fourteen days.

The observations made during the progress of the experiments were as follows.

(*a*) *The effect of the addition of sulphates on the changes induced during the heating of the sera.*

In all cases it was evident that the addition of sulphates increased the precipitation of heat-denaturated protein.

With the aid of the Zeiss immersion refractometer determinations were made of the actual precipitation of protein from the heated sera and also of the increased heat-denaturation induced in the presence of the respective sulphates. The results which were compared with those obtained from control experiments with the original plasma have been embodied in Table III.

TABLE III. *Showing the changes induced during the heating of sera containing sulphates.*

Percentage of electrolyte added to the plasma previous to its being heated	PH of the plasma previous to its being heated	Appearance of the plasma to which the electrolyte has been added (a) Before being heated	(b) After being heated	Percentage increased precipitation of the serum proteins during the heating of the plasma	Percentage increased precipitability of the serum proteins by 30 per cent. of saturation with ammonium sulphate induced during the heating
$MgSO_4 7H_2O$					
0	7·4	clear	opalescent and slight deposit	2·0	17·7
15	7·4	,,	slight precipitate	2·7	17·0
20	7·4	slight precipitate	increased precipitation	3·7	17·7
30	7·4	,, ,,	,, ,,	13·0	24
20 and ammonia	8·0	,, ,,	thin emulsion	28	32·8
K_2SO_4					
0	7·0	clear	opalescent and slight deposit	0	10
5	7·0	very slight precipitate	very slight precipitation	1	11
10	7·0	slight precipitate	precipitation more marked	0	10
15	7·0	,, ,,	,, ,, ,,	3	13
10 and ammonia	8·3	,, ,,	almost solid	36	40
Na_2SO_4					
0	7·0	clear	opalescent and slight deposit	0	10
5	7·0	slight precipitate	slight precipitation	2	10·6
10	7·0	,, ,,	precipitation more marked	2	10
15	7·0	thick ,,	thick precipitate	15	—
10 and ammonia	8·3	slight ,,	,, ,,	33	38
$ZnSO_4 7H_2O$					
0	7·4	clear	opalescent	0	17·7
15	more acid than p_H 4·5	thick precipitate	almost solid	protein completely precipitated during the heating	
20	,,	,, ,,	,, ,,	,, ,, ,,	,,
$(NH_4)_2SO_4$					
0	6·8	thin suspension	thin suspension	0	—
30 saturation	6·8	,,	precipitation increased	10	—
30 ,,	7·1	,,	,, ,,	12	—
30 ,,	7·6	,,	,, ,,	20	—
30 and 0·3 cresylic acid	7·2	,,	,, ,,	30	—
30 and ammonia	8·5	,,	,, ,,	44	—

In those cases where the sulphates of ammonium, potassium, magnesium or sodium had been used, the determination of the refractive indices of the heated and unheated sera of which the [H·] lay between P_H 5·5 and 7·6, showed that the increased precipitation was due to an agglutination of colloidal particles of suspensoid protein into a suspension which ultimately settled as a perceptible precipitate. During the heating there had been no conversion of protein from the emulsoid to the suspensoid condition beyond that experienced in the control sera.

Where the reaction of the plasma had been more alkaline than P_H 8·0 the consistency of those sera containing sulphates in the proportions given in the Table, was invariably changed to that of a thick suspension; in the control experiments with plasma adjusted to the same [H·] the heated liquids remained clear. In these cases the determination of the refractive indices of the sera containing sulphates showed that, during the heating, there had been an actual conversion of the emulsoid protein into a suspension. In the control experiments the changes induced had been insufficient to effect this conversion; the extent of the changes was detected by an estimation of the increased precipitability of the proteins.

From the data furnished with regard to the increased precipitability of the serum proteins by ammonium sulphate it will be seen that, except at concentrations of the electrolyte nearing those required for the precipitation of one or other of the serum proteins, the extent of the heat-denaturation was not directly affected by the addition of those sulphates which caused no appreciable change in the [H·] of the plasma.

Thus, the extent of the heat-denaturation induced in plasma containing the sulphates of ammonium, potassium, magnesium or sodium, was practically the same as in the control experiments and was obviously regulated by the [H·] of the plasma.

The addition of 15 and 25 % of zinc sulphate to the plasma changed the [H·] from a value P_H 7·4 to values more acid than P_H 4·5. As was to be expected, there was complete precipitation of the serum proteins during the heating of plasma to which this sulphate had been added.

It is interesting to note that in the case of sera more alkaline than P_H 8·0 the increased precipitation of protein from the heated sera was approximately a measure of the extent of the heat-denaturation induced; the latter was in no way increased beyond that of the control sera adjusted to the same [H·]. The addition of sulphates to these alkaline sera had thus induced a precipitation of heat-denaturated protein similar to that observed during the

heating of acid sera, or of sera to which either phenol and its homologues, or ether or chloroform had been added.

The results so far obtained show that while the addition of sulphates to the plasma does not directly affect the extent of the heat-denaturation of the serum proteins, it considerably improves the aggregation of suspensoid particles of protein into a filterable form. Moreover, in alkaline sera showing a sufficiently high denaturation, they induce the precipitation of the heat-denaturated protein which would otherwise have remained in the disperse phase.

(*b*) *The increased production of proteins insoluble in brine during the heating of sera containing sulphates.*

Samples of the unheated and heated sera of which the reaction lay between P_H 6·6 and 7·6 were filtered. To a fixed volume of each was added twice the volume of a saturated solution of salt and sufficient solid salt to ensure saturation. From comparative tests it was seen that, in all cases, the amount of "brine-insoluble" protein was considerably increased during the heating of the sera; the increase was more marked in those sera containing sulphates than in the controls with the original plasma.

In the preceding sections of the paper it has been shown that the addition of acid or of trikresol to the plasma increased the production of "salt-insoluble" protein during the heating of the plasma; under certain conditions, the "salting out" of the thus denaturated proteins ceased to be of a reversible nature.

It has been established that sulphates also favour an increased precipitation of heat-denaturated protein by salt. In this case, however, the following evidence shows that in sera of which the reaction was of the order of P_H 7·8, the increased salting out of the denaturated proteins, presumably the result of an electrical charging of the protein molecules by the sulphions, can be reversed, to a certain extent, by sufficiently reducing the active mass of the latter.

A precipitate of heat-denaturated pseudoglobulin, which in the control sera was "brine-soluble," but which had been rendered insoluble in brine by the presence of sulphates, was suspended in a volume of brine twice that of the original serum taken for concentration. After several weeks' extraction it was found that not more than a trace of the protein had been redissolved.

The volume of the brine was then increased to eight times that of the original serum. After a week the amount of protein in solution was not

visibly improved; after three weeks there was an appreciable improvement; by the end of three months a considerable amount had been dissolved, although, even after five months, the recovery was not complete.

The same phenomena are exhibited by the proteins precipitated by the addition of ammonium sulphate in excess of that required to precipitate "salt-soluble" heat-denaturated pseudoglobulin from solution. The ease with which the heat-denaturated pseudoglobulin, thus precipitated, is redissolved in brine depends partly on the extent to which the protein has been denaturated during the heating, and partly on the percentage of sulphate present. Thus, in a case under investigation, while the denaturated pseudoglobulin and antitoxin attached thereto precipitated by the addition of ammonium sulphate to the extent of 30 % of saturation, was readily dissolved by brine, that precipitated from the same solution by the addition of ammonium sulphate in greater amounts was not completely redissolved after six months' extraction with a large volume of brine. Moreover, it was found that, as the heat-denaturation was increased, so the reversibility of this precipitation was either diminished or delayed. Experimental work is in progress to settle this point.

(c) *The percentage removal of the serum proteins and the degree of concentration experienced during the concentration of sera containing sulphates.*

The data with regard to the percentage removal of the serum proteins and the increase in potency obtained during the process of concentration have been recorded in Table IV. A study of the Table shows that in those cases in which the percentage removal of the serum proteins had been greater than 50 % there were appreciable losses of antitoxin. Without doubt the losses were due to the increased insolubility of heat-denaturated protein and associated antitoxin in brine. For the recovery of the antitoxin thus precipitated it was necessary to submit the precipitates to a prolonged extraction in a large volume of brine.

In all cases the end products were more deeply coloured and, generally speaking, neither the percentage removal of the serum proteins nor the degree of concentration experienced was in any way better than that obtained in the preceding sections.

It was subsequently shown that the failure of the sulphates to improve the degree of concentration, in spite of the increase in "salt-insoluble" proteins which they favour, was due to the increased precipitation of albumin at the stage at which pseudoglobulin and antitoxin are precipitated.

TABLE IV.

Showing the results obtained from the concentration of sera by the direct treatment of the sera, heat-denaturated in presence of sulphates, with brine.

Electrolyte added to the plasma previous to its being heated	P_H of the plasma	Percentage denaturation of the serum proteins induced during the heating of the plasma	Percentage removal of the total proteins of the original serum induced during the process of concentration	Percentage of the total antitoxic units of the original serum appearing in the residues from the dialysis of the protein precipitated by the addition of acetic acid to the filtered brined sera	Increased potency per cc. shown by the end product, calculated for purposes of comparison on the basis that they contain 20 % of protein
Controls —	7·0	10	27	—	—
Controls —	7·4	18	35	—	—
Controls —	7·6	28	48	> 90	4
$(NH_4)_2SO_4$ (30 % of saturation)	6·8	10	35	> 85	3·5
do.	7·2	10	35	> 85	3·4
do	7·4	20	48	88	4
do.	8·3	30	51·8	70	4
$MgSO_4 7H_2O$ (30 %)	7·2	13·5	60	70	5·9
Na_2SO_4 (10 %) and 0·3 % cresylic acid	7·6	40	57	70	5
Na_2SO_4 (7 %)	7·4	18	42·6	—	—

IV. THE INVESTIGATION OF THE REASON FOR THE LOWER DEGREE OF CONCENTRATION OBTAINED FROM THE "SALTING OUT" OF THE HEATED SERA WITH SODIUM CHLORIDE THAN FROM THE USE OF AMMONIUM SULPHATE.

Experimental work was next undertaken to ascertain the cause underlying the lower degree of concentration obtained by the direct treatment of the heated sera with salt as in sections I, II and III, than that which is usually obtained as a result of their fractional precipitation with ammonium sulphate.

The concentration of volumes of antitoxic sera, ranging from one to sixty litres, were carried out by the method under discussion and by the Homer [1916, 1918] method. A comparison was made between the respective end products as regards their protein content, the increased potency over that of the original serum and their antitoxic content. The results of the investigation have been embodied in Table V.

The use of ammonium sulphate as the salting out agent in the latter method resulted in end products which showed a potency per cc. about eight times that of the original serum; their protein content indicated that there had been a removal of about 75 % of the serum proteins during the process of concentration.

In the concentration of sera by the former method in which sodium chloride was used as the "salting out" agent the maximum increased

TABLE V. *A comparison between the composition of the end products obtained from the concentration of sera by the methods described in sections I, II and III of this paper and by the Homer* [1916, 1918] *method.*

Series No.	Substances added to the plasma previous to its being heated	P_H of the plasma previous to its being heated	Method of concentration adopted	Percentage of the total antitoxic units of the original plasma appearing in the end product	Increased potency per cc. shown by the end products, calculated for purposes of comparison on the basis that they contain 20 % of protein	Percentage removal of the serum proteins induced during the process of concentration	Percentage of *albumin* admixed with pseudo globulin in the end product
Antitetanic sera							
57	—	7·6	Homer [1916]	> 90*	8·5	73·5	1·5
57	acetic acid	5·05	the method given in section I	88	5	55	21·0
57	ammonia	8·30	„ „ „	88	5	54	22·0
87	ammonia	8·3	Homer [1918]	circ. 85*	8	78·5	2·0
80	0·30 % cresylic acid	7·2	„	90	12	78·0	5·0
85	0·30 % „	7·1	„	circ. 85*	7	78·3	5·0
85	$(NH_4)_2SO_4$ (30 % of saturation)	6·8	method given in section III	> 85*	3·5	35	36·5
85	„ „ „	7·2	„ „ „	> 82*	3·4	35	33·5
85	30 % $MgSO_4 7H_2O$	7·2	„ „ „	70	5·9	60	38·0
85	10 % Na_2SO_4 and 0·30 % cresylic acid	7·2	„ „ „	70	5	57	50·0
85	7·5 % Na_2SO_4	7·2	„ „ „	—*	—	42·6	40·0
Antidiphtheritic sera							
55	0·30 % cresylic acid	7·6	Homer [1918]	circ. 88*	8	78·0	4·9
55	0·30 % „	7·6	method given in section II	88	4	51·0	35·5
55	ammonia	8·3	„ „ „	88	4	46·3	23·5
55	0·30 % cresylic acid and ammonia	8·3	„ „ „	88	4	46·8	35·5
73	$(NH_4)_2SO_4$ (30 % of saturation)	7·1	Homer [1916]	88	10·5	71·0	—
73	„ „ „	7·1	method given in section III	> 90*	4	48·0	25
73	$(NH_4)_2SO_4$ (30 % and 0·30 % of cresylic acid	7·1	„ „ „	70	4	51·8	50·3

* Not tested further because of the lack of experimental animals.

potency that was obtained without undue loss of antitoxin was of the order of five times; no improvement in this respect was effected by the addition of substances such as cresylic acid which are of proven value in the methods involving the use of ammonium sulphate.

It has been shown that cresylic acid materially improves the latter processes by inducing a greater removal of the serum proteins. However, in the method under consideration, the use of cresylic does not appreciably reduce the percentage of the original proteins appearing in the end product.

It was noticed that the end products from the brining of the heated sera were, without exception, more intensely coloured than those now obtained by the Banzhaf and the Homer methods. The colour was invariably most pronounced where the heat-denaturation has been conducted in presence of cresylic acid or sulphates.

It has been recently shown that in the Banzhaf and the Homer methods the colour of the end products depends to a large extent on the presence of heat-denaturated albumin and adsorbed colouring matter of the serum [Homer, 1917, 3, 5]. These observations together with those on the failure of cresylic acid and of sulphates to improve the percentage removal of the serum proteins during the process of concentration, led me to conclude that the end products from the direct brining of sera contain albumin in considerable amounts.

Experimental evidence in justification of this conclusion was furnished in the following way.

Albumin and pseudoglobulin were respectively precipitated from unheated plasma in the usual way. The precipitated proteins were twice redissolved and reprecipitated in order to free them as much as possible from admixture with the other protein entities of the serum. The precipitates after filtration were drained, pressed and dialysed in the usual way. The residues from dialysis were diluted so as to contain about 5 % of protein in solution.

It was found that there was no precipitation of protein from aqueous and from 0·85 % salt solutions of pseudoglobulin and of albumin respectively, of which the [H·] had been adjusted to values between P_H 7·0 and 3·0.

After the addition of electrolytes to the solutions, the precipitation of protein was readily induced at a [H·] between P_H 4 and 5. Thus, the solution of pseudoglobulin saturated with salt began to show slight precipitation on the addition of 0·05 % of glacial acetic acid (circ. P_H 5·5); the precipitation was complete after the addition of 0·30 % of the acid (circ. P_H 4·7). The solution of albumin, similarly treated, showed a slight precipitation on the

addition of 0·15 % of glacial acetic acid (circ. P_H 5·0); while the precipitation was considerable at 0·3 % it was not complete until 0·5 % of glacial acetic acid had been added.

From these observations it follows that the protein precipitated by the addition of 0·25 % of glacial acetic acid to the filtered brined sera contains a considerable amount of albumin admixed with the pseudoglobulin.

Confirmatory evidence on this point was obtained by a gravimetric determination of the proportion of albumin to pseudoglobulin in the respective end products.

The results of the determinations have been included in Table V. They show that, where the [H˙] had been suitably adjusted to the desired value, the proteins appearing in the end product from the concentration of sera by the Homer [1916, 1918] method consisted of about ninety-eight parts of pseudoglobulin to two parts of albumin: where the alternative treatment of the plasma had been adopted, viz., the addition of 0·3 % of cresylic acid to the plasma previous to its being heated, the proportion of albumin rose to 5 %.

The protein appearing in the end products from the methods involving the brining of the heated sera contained a relatively greater amount of albumin; which latter depended on the extent of the heat-denaturation and on the nature of the substances added to the serum previous to its being heated.

In those cases in which the heat-denaturation had been regulated by an adjustment of the [H˙] to values between P_H 5·0 and 8·7, 20 to 25 % of the protein appearing in the end product consisted of albumin. Where the denaturation had been induced in presence of cresylic acid or of sulphates the proportion of albumin was considerably increased, in some cases being as high as 50 %.

The evidence thus adduced shows that, by the direct treatment of the heated sera with salt and the subsequent precipitation of the brined extracts with acetic acid, the degree of concentration cannot be so great as that attained by the use of methods in which the separation of the albumin is more or less assured.

From a study of the data given in Table V it will be seen that the best results were obtained from the brining of those sera in which, previous to their being heated, the reaction had been adjusted to about P_H 8·3.

The addition of electrolytes or of cresylic acid to the plasma adjusted in this way was of no material advantage, for while they induced an increased precipitability of heat-denaturated pseudoglobulin, the advantage thus gained

was more than counterbalanced by the increased precipitability of heat-denaturated albumin.

In fact, for this reason, the addition of cresylic acid or of sulphates sometimes induced a lower degree of concentration than would otherwise have been obtained. Thus in experiments (not recorded in the Tables) where a high percentage removal of the serum proteins had been induced, the end products showed a potency of only 2–3 times that of the original serum; they also contained less than 50 % of the antitoxin taken for concentration.

The low degree of concentration was found to be due to the high percentage admixture of denaturated albumin with pseudoglobulin in the end product; the high percentage loss of antitoxin was due to its precipitation with the "brine-insoluble" heat-denaturated pseudoglobulin.

Summary.

1. The concentration of antitoxic sera can be successfully conducted by a regulation of the heat-denaturation of the serum proteins followed by a direct treatment of the heated sera with brine and salt.

2. The end products thus obtained were clear and readily filterable, as they were not liable to contain the colloidal suspension of heat-denaturated protein which is so often found in those resulting from methods involving the use of ammonium sulphate.

3. Where the heat-denaturation had been of the order of 30 to 40 %, the use of this method led to the removal of about 50 % of the serum proteins. The potency of the end products was about five times that of the original serum.

4. The degree of concentration was not improved by a further increase in the extent of the heat-denaturation or by the addition of substances such as cresylic acid or sulphates to the serum previous to its being heated.

It is a matter of experience that the factors which increased the insolubility of the heat-denaturated pseudoglobulin in brine also increased the precipitation of heat-denaturated albumin at the stage at which the pseudoglobulin and antitoxin are precipitated.

5. In all cases the end products were more deeply coloured and contained a greater percentage of albumin than is found in those now obtained by the use of recent modifications of the Banzhaf [1913] and the Homer [1916] methods.

6. Under some conditions the heat-denaturated proteins precipitated

during the heating of sera containing cresylic acid or of which the reaction is more acid than P_H 5·0, do not redissolve in brine, even on long standing.

On the other hand there is evidence that the proteins precipitated during the heating of sera containing electrolytes can be redissolved by the prolonged treatment of the precipitates with a large volume of brine.

7. For the successful concentration of sera, the precipitation of those proteins to which the antitoxin is attached must not be changed from a reversible to a non-reversible type of reaction. In the latter case, even though the animal tests show that there has been no inactivation of the antitoxin attached to the insoluble particles of protein, from the point of view of the concentration of serum for clinical use this antitoxin is hopelessly lost until a method of dealing with the insoluble precipitates is forthcoming.

8. The method under discussion has not furnished end products showing so great a percentage removal of the serum proteins nor a degree of concentration so high as that now obtained in these laboratories by the Banzhaf and the Homer methods. Nevertheless the description of the method with sodium chloride may be of value to those engaged on problems connected with the routine concentration of sera, for, should untoward circumstances arise to prevent the concentration of sera by the methods now in use, there is hereby furnished an alternative method of proven practicability.

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XVIII. ON THE BIOCHEMICAL MECHANISM OF GROWTH. THE EFFECT OF SODIUM AND CALCIUM IONS ON THE GROWTH OF A TRANSPLANTABLE MOUSE CARCINOMA.

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THIS paper contains an account of experiments in which rapidly growing mammalian cells, namely the cells of a transplantable mouse carcinoma, were exposed in vitro to the action of equimolecular solutions of sodium chloride and calcium chloride with the object of studying the resultant effect on their rate of growth.

The experimental details were as follows. A rapidly growing tumour strain—No. 63 of the Imperial Cancer Research Fund—was selected, which, when made into an emulsion and transplanted in measured doses with a syringe, grows evenly and in a high percentage (90–100 %) of the inoculated animals. A tumour, after being freed as much as possible from all necrotic tissue, was made with scissors into a fine emulsion. If one tumour did not yield sufficient material two or three tumours from the same generation were used and made into one emulsion. One portion of the tumour emulsion was transplanted directly into mice by means of the transplanting syringe in doses of 0·02 cc. This series served as a control. Approximately equal portions of the emulsion, amounting to about 0·5 cc., were transferred by means of the transplanting syringe to two centrifugal tubes. These tubes had a capacity of about 25 cc. and could be closed by means of a rubber washer and a metal screw cap. Equal volumes (about 12 cc.) of sterile $M/7{\cdot}5$ solutions of sodium chloride and calcium chloride in tap water were added to the two centrifuge tubes, so that the latter were about half filled. The tubes with the tumour cells suspended in the two salt solutions were then shaken gently in a shaking machine at room temperature for periods varying in different experiments from 70 minutes to 110 minutes. Since the tubes were filled to only half their

capacity an aeration of the solution was maintained throughout this period. The suspensions were then centrifuged at a moderate speed for about three minutes, and the supernatant fluids, which contained a certain amount of protein, derived presumably from plasma and some necrotic tissue which had remained, decanted. The two masses of cells which had collected at the bottom of the two tubes were shaken out into two glass capsules and cut up again with scissors into an emulsion. The two emulsions were then transplanted by means of the transplanting syringe in doses of 0·02 cc. into two batches of mice. Aseptic conditions were observed throughout the experiment. The subsequent growth was then charted in the usual way in weekly intervals.

The experiments gave a uniform result in showing a distinct inhibition of the growth of those cells which had been treated with calcium chloride. This inhibition manifests itself both in a diminution of the number of positive inoculations and also in a lessened rate of growth of those tumours which had taken. This latter effect is only transient, as will be discussed presently. The cells which had been treated with sodium chloride gave the same high percentage of positive inoculations as the control batch and grew almost as rapidly as the control.

This result naturally raised the further question whether the inhibitory effect of calcium ions could be antagonised by sodium ions. This was tested by adding to the three series of inoculations used in the experiment which has just been described, a fourth series of inoculations in which the cells, after having been suspended in a calcium chloride solution, were further treated with a sodium chloride solution. This experiment was carried out in the same manner as the experiment detailed above, except that three centrifuge tubes were charged with equal portions of a tumour emulsion. Two of these tubes received a $M/7{\cdot}5$ $CaCl_2$ solution. After shaking these three suspensions for equal periods, centrifuging and decanting, the cells contained in one of the two $CaCl_2$ tubes were transplanted at once, as well as the cells which had been treated with sodium chloride solution. To the cells in the second $CaCl_2$ tube fresh $M/7{\cdot}5$ NaCl solution was added, in which they were suspended and shaken for a period of time approximately equal to that during which they had been exposed to the $CaCl_2$ solution. They were then centrifuged, made into an emulsion with scissors and transplanted in equal doses of 0·02 cc. The cells which had been treated in this way took again in 100 % of cases and showed a rate of growth almost, although not quite, as high as that of the cells subjected to the influence of the Na ions alone. This removal of the inhibition induced by Ca ions is all the more striking when one takes into consideration

that the experimental manipulations and more especially the length of time during which the cells are being kept outside the body, are in themselves conditions not favourable to their subsequent growth. These experiments establish therefore clearly the existence of an antagonism between Na and Ca ions with reference to growth.

Fig. 1 which reproduces the chart of such an experiment illustrates the effects produced. The first column (mice Nos. 1–6) represents the growth of the control series, the second column (mice Nos. 7–14) the growth of cells treated with NaCl solution for 75 minutes, the third column (mice Nos. 15–24) the growth of cells treated with $CaCl_2$ solution for the same length of time, and the last column (mice Nos. 25–34) the growth of cells treated first with $CaCl_2$ solution for 75 minutes and then with NaCl solution for 45 minutes.

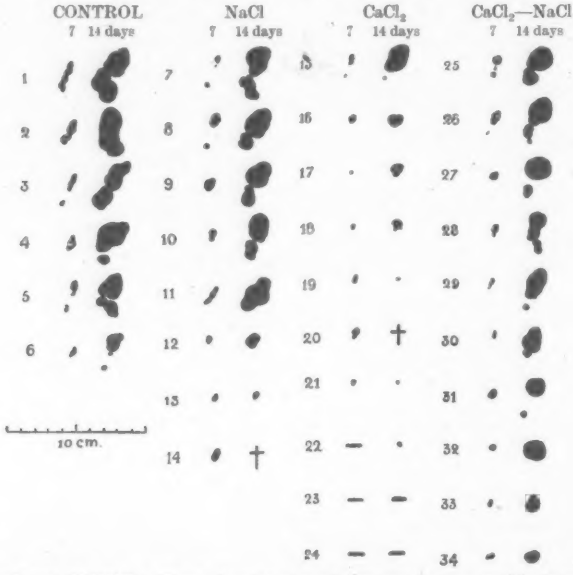

Fig. 1. Experiment 63/170 B. Chart showing size of tumours in two weekly intervals after transplantation. † indicates death of the animal from intercurrent disease; — indicates complete absence of tumour growth. For details of experiment see text.

In this experiment all the animals were killed after the second charting had been made and the tumours weighed. The results are given in Table I.

Incidentally the demonstration of this antagonism disposes of the possibility of attributing the diminished growth on the part of the cells treated with Ca ions to a merely lethal effect which kills off a number of cells and thus greatly reduces the number of living cells introduced with each dose.

TABLE I.

Control		Na ions		Ca ions		Na ions after Ca ions	
No. of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g.	No. of mouse	Wt of tumour in g
1	3·7	7	1·9	15	1·1	25	2·4
2	3·8	8	1·9	16	0·7	26	1·5
3	2·7	9	1·9	17	0·3	27	1·6
4	2·8	10	2·0	18	0·3	28	1·3
5	2·0	11	1·8	19	below 0·1	29	1·1
6	1·2	12	0·6	20	†	30	0·8
—	—	13	0·2	21	below 0·1	31	0·9
—	—	14	†	22	0·2	32	0·9
—	—	—	—	23	—	33	0·7
—	—	—	—	24	—	34	0·5
Total number of mice alive after 14 days	6		7		9		10
Total weight of tumour growth in batch in g.	16·2		10·3		2·6		11·7
Average weight of tumour per mouse in g.	2·7		1·5		0·3		1·2

The inhibition of growth produced by Ca ions in these experiments is, as stated above, transient. If the tumours of the Ca strain are allowed to grow for more than two weeks they resume the rapid growth characteristic of the strain 63 from which they are derived. Indeed some of the tumours show a fairly rapid rate of growth already in the second week after inoculation. Similarly if the tumours of the Ca strain be reinoculated after two weeks without further experimental interference into fresh mice, these inoculations take again in 100 % of cases and grow as well as, if not better than, inoculations made from the control batch or from the Na strains.

It was of interest to determine what would happen if the inhibitory effect of Ca ions were applied repeatedly. Would the growing cells respond again and again to the action of Ca ions? Or would they get accustomed to it and fail to be inhibited in their growth? Or—a third possibility—would the inhibitory effect become more intense and perhaps permanent? In order to test these questions tumour cells were treated with a solution of calcium chloride as described above and inoculated. After two weeks some of the tumours obtained from these cells were made into an emulsion, of which one portion was inoculated at once while another portion was again subjected to the influence of Ca ions and then inoculated. This experiment was continued through five generations. The result eventually obtained in the fifth generation is shown in Fig. 2, which might represent equally well the results obtained in any of the preceding generations: in every case the control batch showed

typical rapid growth in 100 % of inoculations and the Ca batch a transient inhibition, which did not appear to be either less or more distinct than the effect obtained when the treatment with $CaCl_2$ solution was applied for the first time. Nor was it possible to detect any histological difference between the tumours which had been subjected five times to the effect of Ca ions and the tumours of strain 63 obtained in the ordinary course of its propagation.

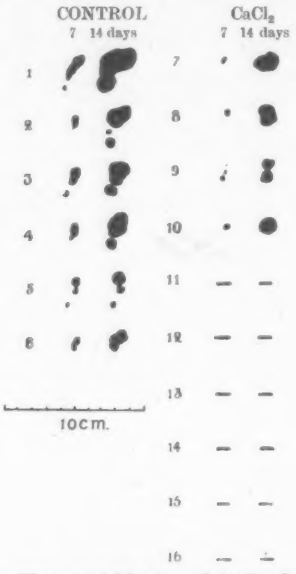

Fig. 2. Experiment 63/172 B. The material for transplantation had been taken from a strain which had been subjected at four successive transplantations to treatment with $CaCl_2$ solution. Part of the material has been transplanted directly ("control series"), another part has been treated again with $CaCl_2$ solution ("$CaCl_2$ series").

There is, however, a distinct difference recognisable both histologically and chemically, when the cells are examined immediately after the treatment with the various salt solutions. Histologically the protoplasm of the cells treated with $CaCl_2$ solution appears denser and less spongy than that of the cells treated with NaCl solution, or of the cells which have received no treatment at all. The dense protoplasm of the cells treated with $CaCl_2$ solution becomes looser again when these cells are treated subsequently with NaCl solution. These morphological differences have corresponding chemical differences in the water content of the cells, which is highest in the cells subjected to the influence of Na ions and lowest in the cells treated with $CaCl_2$ solution. In determining the water content the cells were suspended in the various salt

solutions as has been described at the beginning of this paper. The suspensions were then centrifugalised, the mass of cells at the bottom of the tubes shaken out of the tubes and allowed to fall on blotting paper and the adherent fluid removed by touching the mass of cells gently with blotting paper. The cells were then transferred to weighing glasses and dried at 107° to constant weight. The results of two such experiments are given in the following table.

Water Content of Cells treated with:

Na ions	Ca ions	Na ions after Ca ions
85·4 %	83·8 %	86·8 %
85·1 %	84·0 %	85·2 %

These figures are probably slightly higher than the actual water content, since in removing the adherent water by means of blotting paper only the surface of the cake of cells can be dried and a certain amount of water which lies in the interstices between the cells always remains. A rough estimate of the amount of water thus retained is obtained when the cells are made into an emulsion and drawn up into the transplanting syringe for transplantation. The fluid then rises to the top of the emulsion. It is safe to say that the amount of this fluid is certainly not greater in the case of the cells treated with Na ions than with the cells treated with Ca ions, so that the comparative value of the estimations of the water content is not affected.

It is necessary to consider briefly to what extent the special experimental conditions may have contributed to the effect on growth observed in these experiments. There is firstly the fact that the cells treated with salt solutions were subjected to a greater number of mechanical manipulations and were kept for almost two hours at room temperature outside the body as compared with the control batch which was transplanted immediately. This factor operated evenly in the NaCl and $CaCl_2$ series, so that it cannot have contributed to the difference observed between these two series. Any influence which this factor may have had ought to have come out most clearly in the series in which the inhibitory effect of the Ca ions was antagonised by subsequent treatment with Na ions. It has been pointed out already that the restitution of the rate of growth in this series to the rate observed in the NaCl series adds to the conclusiveness with which the existence of an antagonism between the Na ions and Ca ions could be demonstrated. A second factor is the variation in the actual dose of cells introduced in the different series at each transplantation. After treatment with the various salt solutions the emulsions when taken up in the transplanting syringe were less coherent and looser than the emulsion used in the control series. Moreover, as already

stated, they could not be freed entirely from a small amount of fluid which had remained between the cells after centrifugalisation. As transplantation proceeded and the emulsions were being pressed through the narrow transplanting needle they were compressed and became denser. Although the dosage by volume was kept constant at 0·02 cc., the actual dose of cells delivered each time must have been affected by this factor. In order to determine the degree of this variation the actual amounts delivered were weighed in a separate experiment. It was found that with the emulsion of the control series the amounts remained fairly even throughout a transplantation of fifteen doses, the actual amounts varying from 0·017 g. to 0·022 g. With the emulsion obtained after treatment with the various salt solutions, the first doses amounted to about 0·012 g. and gradually increased to 0·020 g. The NaCl series and the $CaCl_2$ series behaved in this respect in essentially the same manner. It is evident, therefore, that the differences in growth observed between the cells treated with the various salt solutions are not attributable to this factor. It may be partly responsible for the slightly more rapid growth of the control batch as compared with the series of cells treated with NaCl solution (either alone or after a previous treatment with $CaCl_2$ solution). For by varying, in a special experiment, the doses used for transplantation it was found that diminishing the dose of the control mice to 0·01 cc. slightly diminishes the initial growth during the first week but it does not diminish the percentage of positive inoculations, nor does an increase in the dose of cells treated with Ca ions to 0·03 cc. increase the percentage of successful inoculations. Moreover, although it slightly increases the initial growth of such cells, a Ca-series transplanted in doses of 0·03 cc. still shows a marked inhibition even if compared with a control series transplanted in doses of 0·01 cc.

General Discussion.

The inhibition of growth by Ca ions in its bearing on the general problem of growth. An interpretation of the inhibition of growth by exposing cells to the action of Na ions and Ca ions can be given on the basis of previous investigations on the biochemical mechanism of growth [Cramer, 1908, 1910, 1916; Cramer and Pringle, 1910, 1, 2; Cramer and Bullock, 1913]. In a series of observations an attempt had been made to determine the property or properties which enable rapidly growing cells to build up protoplasm more quickly than cells which exhibit a much slower rate of growth although living under identical nutritive conditions. The contrast is thus made, not

between normal cells on the one hand and cancerous cells on the other, but between rapidly growing cells whether cancerous or normal (cells of testis, lymph nodes) and slowly growing cells whether cancerous or normal (cells of liver, kidney, muscle). By formulating the problem in this way it becomes restricted to one aspect of the general problem of growth and so kept separate from other aspects of this problem, such as the initiation of growth, or the extraneous conditions necessary for growth, diet for instance. These various aspects of the growth problem may or may not be interdependent. In the present state of our knowledge it has appeared to us advisable for the sake of clearness to treat them as if they were independent of each other. The material with which we have dealt consists of masses of cells differing markedly in their relative rates of growth while living under identical nutritive conditions. The question which we have tried to answer is: how do these various groups of cells maintain these differences in their relative rates of growth?

The answer to that question may be briefly summarised by stating that a rapidly growing cell works more economically in building up new living protoplasm than a slowly growing cell. It does so by using in the formation of new living protoplasm less of the complex organic colloids (especially proteins) and more water. The greater water content of rapidly growing cells is due to a greater imbibition on the part of the protoplasmic colloids.

If this conception of the problem of the biochemical mechanism of growth as a problem of imbibition is correct, it would follow that it should be possible to demonstrate a parallelism between differences in the rate of growth of different groups of cells and differences in the water content of their protoplasm. The existence of such a parallelism has been demonstrated in a previous paper [Cramer, 1916]. And conversely, it should be possible to alter the rate of growth by altering the imbibition of the protoplasmic colloids. This, we believe, is the explanation of the phenomenon recorded in the present paper.

The loss of water on the part of the tumour cells which has been demonstrated in this paper as resulting from exposure to a solution of calcium chloride recalls experiments [J. Loeb, 1899; Thunberg, 1909, 1910; Widmark, 1910, 1 and 2] in which a similar effect has been observed in the case of muscle cells. Reference may also be made to the inhibition by calcium salts of the imbibition produced in living tissues (muscle, eye, lens) and in non-living colloids (fibrin) by acids and alkalies [M. Fischer, 1908; Botazzi, 1911]. The antagonism between Na ions and Ca ions which has been demonstrated in the

present paper with reference to growth holds good also for other activities of living protoplasm.

The inhibitory effect on growth produced by treatment with Ca ions conforms to the law enunciated by J. Loeb [1910] for the muscle cell. Loeb assumes that such a treatment alters within the cell the relative concentration of Na ions and Ca ions, which can be conveniently represented by the quotient C_{Na}/C_{Ca}. He states that a diminution of the value of this quotient produces an inhibitory effect. This holds good, as Loeb himself has shown, for the contraction of various forms of muscle. Thunberg has shown recently that treatment with $CaCl_2$ solution greatly diminishes the respiratory metabolism of the muscle cell.

This similarity between the process of growth and other manifestations of cell life suggests that growth also should be looked upon simply as a property inherent in living protoplasm in the same sense as metabolism, contractility and irritability. This is by no means a new conception. It is emphasised again in this connection, because the conclusions drawn by many recent workers on the growth problem frequently suggest or imply that growth is a property which can be added to or taken away from living protoplasm. Such expressions as cells "losing" or "acquiring" the power of growth suggest that growing cells contain or possess something which is absent from cells which are said to have "ceased to grow," and a similar conception is implied in the term "growth-substance." The subject is too big to be dealt with here in the course of a digression. It has been referred to because many difficulties which at present surround the problem of growth would, we believe, disappear if it were clearly recognised that there is no qualitative difference between cells with regard to growth: that all living cells possess normally the power of growth but in different degree. It is in some respects unfortunate that in most observations on growth the increase in weight (or length) of an entire, highly differentiated animal has been taken as a criterion of growth generally. This would imply as a premise that this increase in weight or length of an animal as a whole is the result of a process of growth which pervades the entire animal. But the increase in weight or length of such an animal is determined almost entirely by the increase in length of the skeleton. The absence of such an increase, which is often taken to be a criterion of an "arrest of growth" in its most general sense, is obviously not necessarily an indication of the absence of growth processes in the numerous other tissues of the animal. A histological inspection of the sexual organs and lymphoid tissues for instance of an adult animal leaves no doubt that processes of growth are actively proceeding in an

animal in which the increase in length of the skeleton has terminated, as the result of the ossification of the epiphiseal cartilages in the long bones.

The inhibition of growth by Ca ions in its bearing on the growth of neoplasms. One of the first results of experimental cancer research was the demonstration of the fact that the growth of cancer cells, although continued and indeed unlimited, is not continuous [Bashford, Murray and Cramer, 1905; Bashford, Murray and Bowen, 1906]. The growth of a neoplasm shows definite fluctuations in the course of its experimental propagation: a certain strain of cancerous cells may at one time grow very rapidly, as indicated both by the percentage of successful inoculations and by the rapid increase in mass of the transplanted cells. Then, if propagation is continued, the percentage of successful inoculations diminishes suddenly, while those cells which have taken will show a very much slower rate of growth or may after an initial growth undergo spontaneous absorption. During such a period of depression of growth a tumour strain may even die out. If it is successfully carried over such a period, it will increase its rate of growth and maintain it for a time, until again a period of depression supervenes. This cyclical process, which is conditioned by factors inherent in the cell, indicates that the cancerous cell is subject to senescence and in that respect resembles the normal cell, but that it differs from the latter in being able to rejuvenate itself spontaneously and thus to escape death.

The significance of this phenomenon lies, apart from its theoretical importance, in the fact that it reveals a phase of diminished vitality in the life of the cancer cell which may serve as a guide in an attempt to find a rational therapeutic of cancer. Since cancer cells are subject to senescence, it is not unreasonable to expect that a knowledge of the factors on which this spontaneous process of sensecence depends may enable us to find conditions which might favour or maintain this process or even induce it. At present nothing is known about the factors which induce this process spontaneously. As to the nature of the process we have shown in a previous paper that the parallelism between rate of growth and degree of imbibition holds good for these fluctuations in the rate of growth in one and the same tumour strain. The phenomenon of the inhibition of growth recorded in this paper might be described as an experimental senescence of cancer cells, which it simulates also in showing the spontaneous rejuvenescence. It is obvious that the conditions by which senescence has been induced in vitro are crude compared with those which determine it in vivo. At present all that can be claimed for this experiment is that it affords an insight into the nature of the processes which lead to a temporary diminution of the vitality of cancerous cells.

Summary and Conclusions.

1. The effect of sodium and calcium ions on growth has been studied by suspending rapidly growing mammalian cells, namely the cells of a transplantable mouse carcinoma, in isosmotic solutions of sodium chloride and calcium chloride for one to two hours. Subsequent transplantation showed a very distinct inhibition of growth on the part of the cells which had been suspended in the calcium chloride solution. The growth of the cells which had been suspended in sodium chloride solution showed, in comparison with the control cells which had been transplanted without any further experimental interference, a slight diminution which is referable in part, if not entirely, to the additional experimental manipulations involved.

2. During the suspension in the calcium chloride solution the cells lose water and their protoplasm acquires a denser appearance.

3. Both the inhibition of growth and the loss of water produced by calcium ions can be antagonised by a subsequent suspension of the cells in sodium chloride solution.

4. The inhibitory effect on growth produced by calcium ions is transient.

5. An explanation of this effect has been given on the basis of previous observations in which it has been shown that the biochemical mechanism by which slowly growing and rapidly growing cells maintain the difference in their rate of growth is due to differences in the degree of imbibition of their protoplasmic colloids.

6. The inhibition of growth produced experimentally and the subsequent recovery therefrom present a similarity to the spontaneous fluctuations in growth which are exhibited normally by tumour cells in the course of their propagation.

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XIX. CREATINE EXCRETION IN RUMINANTS.

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Preliminary.

A considerable amount of evidence has accumulated to show that privation of carbohydrate or interference with its metabolism leads to an excretion of creatine in the urine. Thus Cathcart [1907] observed creatine in the urine of a starving subject, a like observation being made about the same time by Benedict [1907]. Cathcart [1909] later, found that in inanition the output of creatine is unaffected by feeding proteins or fat, but ceases on feeding carbohydrate. Mendel and Rose [1911, 1] caused the creatinuria of starvation in the rabbit to cease by giving dextrose or starch; protein or fat, singly or combined having no effect.

Excretion of creatine has also been shown in pathological conditions, natural or induced, that interfere with the metabolism of carbohydrate, e.g. diabetes mellitus [Krause, 1910], phloridzin poisoning [Taylor and Cathcart, 1910], destruction of carbohydrate in the tissues by injection of sodium selenite [Cathcart and Orr, 1914, 1] and in various conditions affecting the glycogenic function of the liver, e.g. hydrazine poisoning [Macadam, 1913].

Under the condition of privation of carbohydrate or its faulty metabolism, the acetone bodies that appear in the urine give rise to an error in the Folin method of quantitative analysis of creatinine and creatine, the former appearing less and the latter correspondingly greater than the true amounts [Greenwald, 1913; Graham and Poulton, 1914]. In most of the work referred to above this error was not guarded against and the amounts of creatine shown are doubtless exaggerated.

The creatinuria of starvation and of phloridzin poisoning have been reinvestigated, the error of analysis due to acetone bodies being eliminated, and the original finding confirmed, though the creatine is less in amount and later

in appearance than was formerly supposed [Cathcart and Orr, 1914, 2]. Further, the results obtained on rabbits by Mendel and Rose [1911, 1] are not affected by acetone bodies, which do not readily occur in rabbit's urine.

In all these researches the question turned upon the increased excretion of creatine in privation of carbohydrates in subjects that normally excreted no creatine or only small quantities. It was thought that further information might be obtained by experimenting in the opposite direction, i.e. giving a high carbohydrate diet to an animal that normally excretes creatine in large quantities and observing whether any quantitative relationship existed between the metabolism of carbohydrate and the creatine excretion. This method has the distinct advantage of getting rid of complications arising from the acetonuria of carbohydrate privation. To this end the following experiments were undertaken on ruminants (goats and sheep).

Method.

During each experiment the animal was put in a metabolic cage of such construction that the urine could be collected separately from the faeces. At the close of each experiment it was turned out to graze so that it might be in a normal condition at the commencement of the next period. Urine was collected in twenty-four hour periods and examined immediately at the close of the twenty-four hour period of collection. As no catheterisation was practised there was marked variation in the twenty-four hourly excretions. An approximation to the daily secretion is obtained by giving the average daily excretion of each separate period during which the conditions of experiment were constant. As the urine was examined on 142 days this method of presenting the results tends also to brevity and lucidity. The methods of analysis were: total nitrogen—Kjeldahl; creatine and creatinine—Folin.

I. Preliminary experiments.

Creatine has been found in the urine of cattle [Lindsay, 1911]. The figures however are rather scanty and variable. Through the kindness of Dr Leonard Findlay several ruminants were rendered available and some preliminary work was done to ascertain to what extent creatine is normally excreted in ruminants. Table I gives the results obtained: the figures are the average daily amounts excreted for the periods stated.

TABLE I.

Creatine excretion in ruminants.

(Daily averages.)

Animal	Length of exper.	Tot. N g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Diet
He-goat, age 6 mon.	19 days	2·04	0·220	0·198	1·1	Bran, hay, turnips
Sheep, ewe, non-lactating	4 ,,	2·34	0·424	0·196	2·1	Hay and turnips
	4 ,,	?	0·446	0·397	1·1	Hay
Goat A, lactating, non-pregnant	7 ,,	7·61	0·620	0·457	1·4	Hay, oats, bran
	8 ,,	10·30	0·660	0·336	2·0	Hay, turnips, bran

In goat A, the total nitrogen figures include both milk and urine.

In the creatinine excretion of each animal there appeared a certain degree of uniformity not shown in the case of creatine which varied between 40 and 90 % of the amount of creatinine.

The urine was usually alkaline. To ensure that alkalinity of the urine had no influence in altering the proportion of creatinine and creatine several urines—dogs' and human—were treated with inorganic alkalis. No change was detected in the creatine or creatinine content. On nine days in the course of these experiments the urine was acid to litmus paper without any change in the proportion of creatinine or creatine being observed. The loss of creatinine which occurs in alkaline urines appears to be due not to the alkalinity *per se* but to organisms that flourish in an alkaline medium.

To ascertain the average creatine excretion under conditions as nearly normal as possible of goat B, in milk, that chosen for the following experiments, the animal was taken from grazing, put up in the metabolic cage and fed on turnips, oats and hay for five days. It was then let loose to graze for seven days and put up for a further period of nine days on the diet as before. The averages for fourteen days appear in Table II.

TABLE II.

Normal creatine excretion in goat B (lactating).

(Daily averages.)

Length of exper.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Milk cc.	Diet
14 days	5·04	0·85	0·31	2·7	79	Hay, turnips, oats

II. Influence of carbohydrate on creatine excretion.

Experiment I. This experiment was divided into four periods of five days each during which the diet was changed from a high protein and low carbohydrate to a low protein and high carbohydrate diet. The composition of the diets was as detailed below:

Period	Diet	Protein g.	N approx. (prot./6·25) g.	Fat g.	Carbohydrate g.
1	"Plasmon," 250 g. Bean meal 75 g. Turnip 200 g.	200	32	1·6	66
2	Potatoes, 1400 g.	14·98	2·4	1·4	224
3	Potatoes, aver. 1800 g.	19·26	3·1	1·8	288
4	Potatoes, 2200 g.	23·54	3·8	2·2	352

The composition of the potato diets is calculated from the average percentage of digestible nutrients given by Hendry [1913], viz. protein 1·07 %, carbohydrate 16·02 %. The figures given by Kellner [1909] vary as follows: protein 0·9 to 1·3 %; carbohydrate 12·5 to 24·6 %.

The animal was milked daily and the total nitrogen of the milk estimated, so that the combined nitrogen excretion of urine and milk might give some indication of the protein utilised in the periods of low protein diet. Table III gives the averages of the five day periods of the twenty consecutive days.

Table III.

Influence of carbohydrate on creatine excretion. Goat B (lactating).

(Daily averages.)

	Intake		Output					
Period	N approx. g.	Carbohydrate g.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Milk cc.	Change in wt. of goat kilos
1	32	66	28·11	0·91	1·13	0·8	219	loss 0·05
2	2·4	224	5·04	0·80	0·38	2·1	192	,, 2·2
3	3·1	288	4·08	0·98	0·23	4·2	176	,, 0·3
4	3·8	352	2·90	0·93	none	—	81	gain 0·3

Experiment II. In the foregoing experiment the flow of milk decreased from 219 to 81 cc. per day, so that an amount of material formerly drained off from the economy in the milk remained for consumption in the tissues. To obtain results uncomplicated by variations in milk flow this experiment (II) was delayed until lactation had ceased. The course of events during cessation of lactation is recorded in Experiment III. To eliminate any of the effects of the high protein diet being carried over to the subsequent period the animal

was put upon a natural diet of turnips, hay and oats for an initial period of six days. It was then put upon a high carbohydrate diet of potatoes, which for the first period of three days was less than the animal's requirements. The diet was increased as follows:

Period	Length of Period	Diet	Protein g.	N approx. prot./6·25 g.	Fat g.	Carbo-hydrate g.
1	6 days	Turnips, hay, oats		*ad lib.*		
2	5 „	Potatoes, 960 g.	10·3	1·6	1	154
3	3 „	„ 2000 g.	21·4	3·4	2	320
4	3 „	„ *ad lib.*	?	?	?	?

The results are given in Table IV.

TABLE IV.

Influence of carbohydrate on creatine excretion. Goat B (non-lactating).

(Daily averages.)

		Intake		Output				
Period	Length of period	N approx. g.	Carbo-hydrate g.	Tot. N g.	Creatinine g.	Creatine g.	Creatinine Creatine ratio	Change in wt. of goat kilos
1	6 days	?	?	2·30	0·85	0·14	6	loss ·1
2	3 „	1·6	154	3·39	0·90	0·20	4·5	„ 1·6
3	3 „	3·4	320	3·14	0·91	0·18	5	„ ·1
4	3 „	3·4+	320+	2·35	0·92	none	—	„ ·3

The low creatine excretion in the initial period of six days is believed to be due to the fact that the animal had just ceased lactation, the last milk having been drawn off three days before the beginning of the experiment.

III. INFLUENCE OF CESSATION OF LACTATION ON CREATINE EXCRETION.

The following experiment intervenes in time between those recorded in Tables III and IV. The diet consisted of hay, oats and turnips. During the period, the animal was twice put to graze in the open, as a result of which the milk flow tended to return. While in the metabolic cage the flow steadily decreased. During the period the goat increased in weight from 32·7 to 35·4 kilos. The results are recorded in Table V.

RESULTS.

I. CREATINE. (1) *Normal Excretion.* In all the animals put under observation there occurs, except on the high carbohydrate diet, an excretion of relatively large quantities of creatine. In the various animals and on the

various diets the amount fluctuates between rather wide limits. No constancy appears in the creatinine/creatine ratio, which in the preliminary experiments recorded in Table I varies between 2·1 and 1·1.

TABLE V.

Cessation of lactation, goat B.

(Daily averages.)

Length of period	Milk cc.	Tot. N urine + milk g.	Creatinine g.	Creatine g.	Creatinine / Creatine ratio	Remarks
5 days	120	6·12	1·01	0·51	2	Milk flow constant
7 „	154		goat at grass			„ increasing
9 „	55	3·62	0·77	0·28	2·8	„ decreasing
10 „	11		goat at grass			„ increasing
7 „	6	5·33	1·02	0·26	3·9	„ decreasing
7 „	1	2·82	0·88	0·23	3·8	„ „
6 „	nil	2·30	0·85	0·14	6	„ ceased

(2) *Influence of Carbohydrates.* The highest creatine excretion—1·13 g. per day—occurs on the diet with the abnormally low carbohydrate content—66 g. per day. In both Tables III and IV as the carbohydrate content of the diet is increased, the creatine excretion decreases. The close relationship that exists is shown in Table VI.

TABLE VI

	Period	1	2	3	4
Table III	Carbohydrate in diet, g.	66	224	288	352
	Creatine excret., g.	1·13	0·38	0·32	nil
Table IV	Carbohydrate in diet, g.	?	154	320	*ad lib.*
	Creatine excret., g.	0·14	0·20	0·18	nil

The decrease is more rapid in Table III. This may be because the effects of the increased carbohydrate ingestion are augmented by the increased retention of material in the blood due to the reduction in the milk flow, which occurred during the course of the experiment.

(3) *Influence of Cessation of Lactation.* Table V shows a decreased creatine output accompanying the decreased milk flow. The two declines do not run parallel and the greatest decrease in the creatine occurs on the six days after the flow had completely ceased. During the period covered by Table V the animal increased in weight by 2·7 kilos.

(4) *Influence of Proteins.* In periods 2, 3 and 4 an increased nitrogen intake of—in g. per day—2·4, 3·1 and 3·8 coincides with a decreasing creatine output of 0·38, 0·23 and nil respectively: a like decreasing creatine output

occurs simultaneously with an increasing nitrogen intake in Table IV. On the other hand, the highest creatine excretion occurs on the highest protein intake, and in general in both Tables III and IV the decreased creatine excretion is accompanied by a decreased total nitrogen excretion.

In Table III on the fifteen days of periods 2, 3 and 4 the total nitrogen excreted amounts to 60·1 g. while the total nitrogen intake is 46·5 g.—a loss of 13·6 g., indicating a catabolism of about 85 g. of tissue protein. There is no indication of this endogenous protein catabolism producing any addition to the excretion of creatine, which during the period decreases to zero.

II. Creatinine. The excretion of creatinine shows remarkable uniformity, compared with the wide fluctuations shown by the excretion of total nitrogen and of creatine. Thus, in Table I, in the two periods of the ewe the amount, in g. per day, is 0·424 and 0·446 and in goat A 0·62 and 0·66. In goat B the highest is 1·02 and the lowest 0·77.

Taking the preliminary period of goat B, Table II, as the normal, we have 0·85 g. per day in an animal of 35 kilos, i.e. 24 mg. per kilo, a ratio somewhat similar to that found in dogs, and in man, suggesting a similar metabolism.

Discussion of Results.

1. *Production of Creatine.* The fact that in a period of progressively increasing intake of protein of constant composition there occurs a decreasing creatine excretion (Tables III and IV) seems to indicate that the creatine, of that period at least, is not derived from the catabolism of exogenous protein. On the other hand the continuous excretion, in ruminants, of large quantities of creatine on a normal diet, as well as on a creatine-free high protein diet, points to a production of creatine from a source other than the catabolism of endogenous protein.

These results suggest that while quantities of creatine may appear as a consequence of the rapid disintegration of muscle tissue, or of the catabolism of certain exogenous proteins, these are merely irregular and adventitious increments to a constant creatine production in the tissues.

The results provide no means of estimating the amount produced. If, as is suggested below, creatine is utilised, the amount excreted would merely be the surplus of production over utilisation. In the fourteen days' period of goat B, Table II, the daily average on a diet of turnips, oats and hay is 0·31 g. On the lowest carbohydrate diet it is 1·13 g. while on the highest carbohydrate diet the excretion reaches zero. Provided there is no creatine of exogenous

origin, there appears to be the possibility of the production and utilisation of over a gram per day.

On the whole the results would seem to lend support to the view that creatine is produced within the tissues in relatively large amounts, a conclusion arrived at by Benedict and Osterberg [1914], from results obtained on a dog under the influence of phloridzin.

2. *Fate of Creatine.* In so far as the creatine under discussion is being produced within the metabolic field, it is probably in a more favourable position for conversion to creatinine if such takes place, than is creatine given by the mouth or injected subcutaneously. The relatively uniform excretion of creatinine gives no indication of any conversion. If creatinine is derived from creatine the amount converted depends upon some constant metabolic process which is not affected by the amount offered for conversion.

Van Hoogenhuyze and Verploegh [1908] concluded that creatine is usually broken down in the liver. Noël Paton and Mackie [1912] ligatured the portal vein and its radicles in a duck and found no change in the creatine excretion. Towles and Voegtlin [1911] in a dog with an Eck's fistula found no change in the creatine or creatinine excretion. Dakin [1907] and Twort and Mellanby [1913] failed to discover any ferment in the liver capable of acting upon creatine. The influence of the liver in the metabolism of creatine is evidently indirect and possibly solely by virtue of its glycogenic function.

The question as to the fate of creatine cannot be regarded as settled by assuming either its destruction in the liver or its conversion to creatinine.

In the present experiments it has been shown that in an animal normally excreting creatine, the excretion decreases *pari passu* with the addition of carbohydrate to the diet, a result which is the complement of that arrived at by the workers referred to above, viz. the excretion of creatine in carbohydrate privation in an animal normally excreting no creatine. A close relationship therefore appears between the metabolism of carbohydrate and that of creatine. It can be stated at least, that increased carbohydrate ingestion and decreased creatine excretion are in some way causally connected.

Two possible views are considered. (1) Creatine is only produced in the absence of carbohydrate. (2) Creatine or its precursor is constantly being produced but is utilised in the metabolism of carbohydrates.

According to the first view, the excretion of creatine is an indication of the break-down of muscle tissue, and carbohydrate exerts its influence on the excretion by virtue of its presence preventing the necessity for this break-down. In all probability this source supplies at least a portion of the creatine

excreted in starvation, in which the excretion of creatine is in some degree proportional to the break-down of tissue protein [Noël Paton, 1910]. As has been suggested above, however, the quantities of creatine excreted in ruminants cannot wholly be accounted for by the catabolism of endogenous protein, so that this view seemingly fails to cover all the facts as now presented.

The second view—the utilisation of creatine, a conclusion arrived at by Cathcart [1912], from the results of work done in this laboratory, and later suggested by Benedict and Osterberg [1914]—finds support in the fact that on the high carbohydrate and low protein diet, where there is evidence of a break-down of tissue protein and, presumably, the liberation of creatine, there appears in the urine no additional creatine, the excretion of which during the period decreases to zero.

If, as has been suggested, there occurs a constant production of creatine, the decrease in the excretion concomitant with the increased ingestion of carbohydrates points to a utilisation of creatine in the metabolism of carbohydrate.

If this view were correct one would expect to find, that, in cases of excessive consumption of carbohydrate, the creatine in the tissues would be utilised in excess of the production and the stock diminished. Myers and Fine [1913] record a decrease in the percentage of creatine in muscle from 0·520 to 0·482 on feeding on a pure carbohydrate diet. On the other hand, when carbohydrate is not presented for consumption there would be an accumulation and excretion of creatine. Demant [1879] observed an increase in the creatine content of muscle during starvation, an observation confirmed by modern methods by Mendel and Rose [1911, 2], while the excretion of creatine in inanition has been repeatedly observed, *vide supra*.

It may be, that creatine or whatever body represents it in the tissues serves as a connecting link securing the incorporation of the carbohydrate molecule within the protoplasm complex in some glucoside-like combination, in which the catabolism of the carbohydrate moiety occurs.

Conclusions.

1. In ruminants creatine is normally excreted in relatively large quantities.
2. The amount of creatine excreted is in inverse proportion to the amount of carbohydrate in the diet.
3. Cessation of lactation causes a decrease in the amount of creatine excreted.

4. There is a source of creatine other than the catabolism of protein either exogenous or endogenous.

5. Arguments based on the results are advanced in support of the view, that creatine is formed in relatively large quantities, that it is utilised in the tissues and that its utilisation is intimately connected with the metabolism of carbohydrate.

This paper is an extract from a section of a thesis submitted for the M.D. degree of Glasgow University in May 1914.

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Further research on the excretion of creatine on ruminants was being carried out in the summer of 1914. It is hoped to resume the work as soon as an opportunity is afforded.

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XX. AN INVESTIGATION OF THE METHODS EMPLOYED FOR COOKING VEGETABLES, WITH SPECIAL REFERENCE TO THE LOSSES INCURRED. PART I. DRIED LEGUMES.

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The value of dried legumes as articles of diet has long been known, but the present necessity for economising other foodstuffs has led to far more general recognition of the use and importance of these vegetables.

At the request of the Foods Committee of the Ministry of Munitions this investigation was undertaken with a view to obtaining some data as to the losses incurred and the time required for cooking dried vegetables under various conditions.

It was thought that from the information thus gained it would be possible to devise a convenient method of cooking dried legumes on a large scale which, whilst satisfactory in other respects, would minimise as far as possible the waste of food material due to losses in cooking and also reduce the time of cooking, thus effecting a saving in the consumption of fuel.

Some information on the subject of dried legumes is to be found in the bulletins issued by the United States Department of Agriculture [Abel, 1900; *Farmers' Bulletin*, 1903; Wait, 1907].

These publications discuss briefly the cooking of dried legumes but deal chiefly with their nutritive value and digestibility; their scope is therefore somewhat different from that of the present enquiry.

The varieties of dried legumes most commonly used in this country appear to be butter beans, haricot beans, and peas. As the butter beans were found to be rather more uniform in size and shape than the other varieties, most of the experiments were made with these and a few experiments were then made with haricot beans (white and brown) in order to see if similar results were obtained.

A study of the methods usually advocated for cooking dried legumes

seemed to indicate that the subject could be dealt with best in the following sections:

I. The use of tap and distilled water for cooking beans and the effect of soaking previous to cooking.

II. The use of sodium bicarbonate in cooking beans.

III. The use of salt in cooking beans.

IV. The possibility of cooking beans by steaming.

V. Note on haricot beans and peas.

VI. Suggested methods of cooking dried vegetables.

I. The effect of soaking beans previous to cooking and of soaking and cooking in distilled and tap water.

Dried beans are usually soaked in water for at least eight hours previous to cooking. The beans so treated take up water, swell considerably and are generally supposed to cook more rapidly than those which have not been soaked previous to cooking.

The use of soft water in preference to hard water for soaking and cooking beans is also usually advocated since the presence of calcium salts in the water is stated to retard the softening of the beans and prolong the time of cooking[1].

A series of experiments was therefore made in order to compare the time of cooking and the loss in solid matter during cooking of beans both dry and previously soaked and to find how far these results were modified by using distilled water as compared with ordinary London tap water. (Temporary hardness = 12 parts per 100,000. Permanent hardness = 5 parts per 100,000.)

The dry beans were heated in a steam oven to constant weight and were found to contain 12 % of moisture which has to be allowed for in relation to losses incurred in cooking.

The experiments were carried out under conditions which reproduced as far as possible those of the ordinary kitchen. The method adopted for cooking the beans was as follows:

A. Beans cooked without previous soaking.

100 g. of beans were placed in a strainer and washed with cold water in order to remove any adhering particles of dirt. The beans were then placed in an enamel saucepan, 500 cc. of cold water were added, the saucepan was covered with a lid and the temperature of the water was raised to the boiling point and kept at this temperature until the beans were cooked. The time required for cooking was then recorded.

[1] See Robert Hutchison, *Food and the Principles of Dietetics*, also general statements in cookery books.

B. Beans soaked in water previous to cooking.

The beans (100 g.) were washed with cold water as in *A* and were then covered with 250 cc. water and allowed to stand overnight (*i.e.* about 16 hours). The soaking water was strained off, measured and evaporated to dryness and the residue weighed in order to determine the loss in solid matter due to soaking. The beans were then placed in an enamel saucepan and covered with 500 cc. water less the volume of water taken up by the beans in soaking; this was found to be in each case about 100 cc. so that the volume added was about 400 cc. The cooking was then carried out as in *A*.

Cooked beans. The cooked beans were in each case separated as completely as possible from the cooking water by straining, allowed to cool at the room temperature and weighed when cold. This weighing gives the weight of the cooked beans in the condition in which they are ordinarily served. The appearance and taste of the beans were then examined and reported on.

Cooking water. The cooking water was evaporated to dryness and the residue weighed. This residue gives the total solid matter extracted from the beans in cooking together with the residue, if any, from the water used. A correction was made where necessary for such residue.

Since the dry uncooked beans used for the experiments were found to contain 12 % of moisture the residue from the cooking water represents the loss of solid matter on 88 g. of solids, from which figure the percentage loss, *i.e.* the loss on 100 g. of solids, was calculated. The time of cooking, weight of cooked beans and percentage of loss of solid matter in cooking as observed in a number of experiments both with tap and distilled water are given below.

TABLE I. (Tap water.)

A. 100 *g. beans cooked in tap water without previous soaking*			*B.* 100 *g. beans soaked and cooked in tap water*				
Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss of solids
2 h. 10 m.	244	12·5	2 h. 0 m.	231	13·6	1·8	15·4
2 15	223	12·8	2 0	235	8·4	2·2	10·6
2 0	230	—	2 0	—	11·5	1·7	13·2
2 15	—	11·6	1 50	210	8·2	1·5	9·7
2 15	229	12·9					
2 0	230	10·9					
2 15	235	8·5					
2 10	219	8·1					
2 15	237	11·1					
2 0	232	9·3					
2 0	214	9·6					
2 0	211	8·6					
Average 2 h. 5 m.	220·5	10·5	2 h. 0 m.	228	10·4	1·8	12·2

TABLE II. (Distilled water.)

A. 100 *g. beans cooked in distilled water without previous soaking*			*B.* 100 *g. beans soaked and cooked in distilled water*				
Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in cooking	Total percentage loss
1 h. 45 m.	242	—	1 h. 45 m.	231	12·8	2·2	15·0
1 45	218	13·7	1 45	235	9·4	2·2	11·6
1 45	213	11·6	1 45	—	10·7	1·7	12·4
1 45	229	10·7	1 55	208	8·6	—	
2 0	205	8·6					
Average							
1 h. 45 m.	218	11·1	1 h. 45 m.	224	10·4	2·0	12·6

Discussion of results of Tables I and II.

Time of cooking. It will be seen from the results given above that soaking previous to cooking does not reduce the time required for cooking to any very marked extent, but that the time required with distilled water is less than in the case of tap water.

Cooked beans. Soaking beans previous to cooking has practically no effect on the weight of the beans when cooked, the weight of cooked beans being in each case rather more than double that of the raw beans. The soaked beans were a better colour, *i.e.* whiter, and had rather a less pronounced flavour than those which were cooked without previous soaking. The beans cooked in tap water had a better flavour than those cooked in distilled water, which were rather tasteless.

Loss in solid matter. As regards the loss in solid matter during cooking the mean results show that the loss with tap and distilled water is practically the same. Taking into consideration the solid matter extracted by soaking, the total loss in the case of the soaked beans is greater than in the case of those cooked without previous soaking. The individual experiments show considerable variation in the amount of solid matter extracted during cooking; this no doubt is due mainly to the difficulty of determining the exact stage at which the beans are cooked.

The method usually adopted in the kitchen is to prod the beans with a fork and to stop the cooking when the beans have reached what is considered to be the required degree of softness. Since the experiments were to be carried out as far as possible under conditions similar to those of an ordinary kitchen the same method was adopted in the laboratory though it is obviously open to objection.

When the beans are tested the skin is pierced and this will tend to increase the amount of solid matter removed. This is especially the case when the cooking of the beans is nearing completion. The results are given in the order

in which the experiments were carried out and it will be noted that the earlier experiments showed, on the whole, greater losses than the later ones.

This would be accounted for by the fact that at first some difficulty was experienced in deciding when the beans were cooked; they had to be tested several times and there was probably also a tendency towards overcooking; whereas in the later experiments more experience had been gained in judging the end point, the beans were not tested so frequently and the cooking was stopped before the beans began to disintegrate.

It might be thought that the time of cooking would indicate whether the beans had been overcooked or not, but this is not a sufficiently reliable check as owing to the difficulty of exactly regulating the gas supply the time required for cooking was found to vary slightly from day to day and it was noticed that if the cooking was not stopped when a certain stage was reached the beans began to break up rather rapidly and that in the case of peas this tendency was even more marked than in the case of beans.

In order to show the effect of overcooking on the loss in solid matter the beans in one experiment were purposely overcooked, the time allowed being 2 hrs. 25 mins.; the loss in solid matter was then found to be 16·6 % of the total solids.

It would of course be possible to employ a more uniform source of heat and it is hoped to make some experiments under such conditions later.

For the purpose of the present enquiry however it was thought best to adopt such methods as would ordinarily be employed in the kitchen. In dealing with other methods of cooking it was therefore decided that several experiments should be made in each case and an average then taken, and a control experiment with tap water made from time to time. It was hoped that in this way the errors dealt with above might be to some extent eliminated.

Nature of residue. The residues obtained from the soaking and cooking waters have not yet been fully examined and the discussion of their exact nature must be left until some further investigations of the subject have been made.

Residue from soaking water. The residue from the soaking water is dark brown in colour, difficult to dry and if exposed to the air rapidly takes up water and becomes syrupy. The odour and taste is somewhat similar to that of a meat extract. The residue from dry beans soaked in tap water was found to contain 2·3 % of nitrogen probably in the form of extractives and the mineral matter extracted by soaking was 1·54 % of the total mineral matter present in the beans.

Residue from cooking water. The residue from the cooking water is lighter in colour than that from the soaking water and is less deliquescent. The taste and odour are similar but less pronounced.

The residue from dry beans cooked in tap water contains 2·4 % nitrogen corresponding to a loss of 7·5 % of the total protein matter present in the beans and the mineral matter extracted by the cooking is 36·8 % of the total mineral matter present in the beans.

A few experiments were also made to see if there was any advantage to be gained by (1) cooking the beans in the water in which they had been soaked, (2) cooking the beans by the addition of boiling instead of cold water. The results obtained however were very similar to those given above.

In (1) the residue from the cooking water was equal to the combined residue obtained from the soaking and cooking waters in the previous experiments.

In (2) allowing for the time required for bringing the water to the boil the time required for cooking was the same as in the above experiments and the solid matter extracted by cooking was about the same.

II. The use of sodium bicarbonate in cooking dried beans.

Sodium bicarbonate and also washing soda are sometimes added in small quantities to the water in which dried legumes are to be cooked or if they are soaked previous to cooking it may be added to the soaking water.

It is usually stated that this treatment softens the water and so tends to reduce the time of cooking[1].

Although the degree of hardness of the water used for cooking must be taken into account in determining the amount of sodium bicarbonate which should be added it would appear from the experiments now to be described that the alkali has a more specific action than can be accounted for by the above explanation.

In this connection it may be mentioned that Snyder [*Minnesota Station Bulletin*] states as a matter of common observation that when beans are properly prepared with a small amount of soda and salt there is less formation of gas in the intestines during digestion. A series of experiments was therefore made in order to compare the times of cooking and the loss in solid matter when beans were cooked (*A*) with the addition of varying amounts of sodium bicarbonate to the cooking water, (*B*) after soaking in water to which varying amounts of sodium bicarbonate had been added.

[1] See Hutchison, *Food and the Principles of Dietetics*, also general statements in cookery books.

The quantities used, the method of cooking and the subsequent treatment of the cooked beans and cooking water were the same as in the previous experiments.

A. The addition of sodium bicarbonate to the cooking water.

Beans were cooked, without previous soaking, in tap water to which a weighed amount of sodium bicarbonate had been added. The quantity of alkali left in the cooking water at the end of the experiment was estimated and the residue corrected by a corresponding amount. The mean results of these experiments are given below:

TABLE III.

Beans (100 *g.*) *cooked in tap water containing sodium bicarbonate.*

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking
0·05	2 h. 0 m.	221	10·3
0·1	1 50	216	11·3
0·25	1 45	225	14·3
0·5	1 15	240	22·3
1·0	1 0	190	27·3

The results are also shown graphically below. The logarithms of the relative concentrations of sodium bicarbonate used have been plotted instead of the actual concentrations so that equal changes in the concentration may be represented by equal lengths of ordinate.

Graph showing the Loss of Solid Matter compared with the Concentration of Sodium Bicarbonate used.

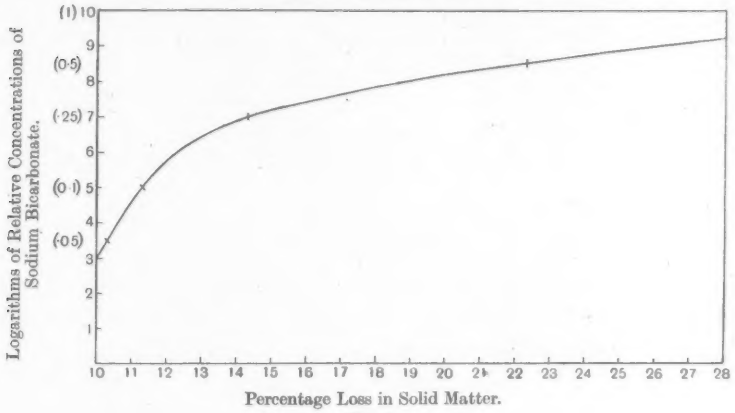

Fig. 1.

Discussion of the results of Table III.

The effect of the addition of sodium bicarbonate both on the time of cooking and on the loss in solid matter is somewhat striking.

The high percentage loss of solids obtained when the sodium bicarbonate added is as high as 0·5–1·0 % is attributable to the fact that under these conditions the beans soften and disintegrate rapidly, hence a large proportion of solid matter passes into the cooking water, and the latter becomes very turbid. The beans become distinctly yellow in colour, are slimy to the touch, and practically uneatable.

Similar effects, though in a much less marked degree, were also observed when 0·25 % sodium bicarbonate was used.

The most satisfactory results (for the particular tap water used) were obtained when 0·1 % of sodium bicarbonate was used.

As shown in the above results, the time of cooking was then the same as the time required with distilled water. The loss of solid matter was only slightly greater than in the case of tap water (Table I), and was slightly less than the total loss observed when the beans were soaked and then cooked in tap water.

There is no advantage to be gained by adding quantities of sodium bicarbonate less than 0·1 %, since the time required with the addition of 0·05 % is the same as when untreated tap water is used (Table I).

More information is needed before any complete explanation of the action of sodium bicarbonate can be attempted, but the solubility of the legumin of the beans in alkali must play an important part, and it is interesting to note that Osborne [1909], in dealing with vegetable proteins, states that "the amount of protein extracted by alkalies is usually greater than that extracted by neutral saline solutions or by water, in fact in many cases it is very much greater. Sufficient attention has not yet been directed to the cause of this difference and for this reason our present knowledge of the character of the total protein constituents of the greater number of seeds which have been studied is incomplete."

B. The addition of sodium bicarbonate to the soaking water.

Beans were soaked overnight in tap water containing varying amounts of sodium bicarbonate. The soaking water was strained off, measured and evaporated to dryness, and the residue weighed. An estimation was made of the quantity of sodium bicarbonate left in the soaking water which was then deducted from the total residue obtained.

The amount of sodium bicarbonate absorbed by the beans during soaking was found, for each concentration of the alkali, to be proportional to the volume of water absorbed which was about 100 cc. in each case.

The average results obtained are shown in Table IV.

TABLE IV.

Beans (100 g.) soaked overnight (about 16 hours) in tap water containing sodium bicarbonate.

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
0·25	1 h. 15 m.	222	10·6	1·8	11·4
0·5	1 15	214	9·2	1·5	10·8
1·0	1 0	211	8·7	1·5	10·8
2·0	1 0	212	13·1	1·5	14·6

TABLE V.

Beans (100 g.) soaked for four hours in water containing sodium bicarbonate.

(Average results.)

Parts of sodium bicarbonate per 100 parts water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
1·0	1 h. 30 m.	222	10·8	0·2	11·0
2·0	1 30	234	12·0	0·2	12·2

Discussion of Tables IV and V.

The time required for cooking the beans after soaking in water containing sodium bicarbonate is considerably less than when either tap or distilled water is used (Tables I and II).

The reduction in time thus effected is fully as great as that observed when sodium bicarbonate up to 1 % is added to the cooking water and the reduction in the former case is not accompanied by the objectionable features noted in the case of the latter. Further experiments show that when 2 % of sodium bicarbonate is used the time of cooking is the same as in the case of 1 %, but the loss in solid matter is greater. The beans also were yellowish in colour and exhibited, though to a less degree, other objectionable features noticed in the case of beans cooked in water containing over 0·2 % of sodium bicarbonate (Table III).

The addition of sodium bicarbonate to the soaking water appears to diminish the amount of solid matter extracted from the beans by soaking, and it was also noted that these residues were lighter in colour than those obtained from tap water (see Section I).

This effect is somewhat curious, but the whole question of the action of sodium bicarbonate needs to be more fully investigated under more carefully controlled conditions and it is hoped that such an investigation may be made at a later date.

Table V shows the average results obtained when the beans are soaked for a shorter period, namely four hours, since it was thought that in some instances this might prove to be a more convenient method. The results show that the reduction in time of cooking thus effected is not so great as when the beans are soaked for a longer period, but the time required is appreciably less than that required with tap or distilled water (Tables I and II).

III. The use of salt in cooking dried legumes.

Vegetables are usually cooked in water containing salt with a view to improving their flavour, but in the case of dried legumes the statement is frequently made in cookery books that salt should not be added to the cooking water, as it tends to harden the skins and prolong the time of cooking and that this difficulty may be avoided by delaying the addition of the salt until the cooking is nearing completion.

It therefore seemed advisable to make a few experiments in order to compare the time of cooking and loss in solid matter when beans were cooked in water containing varying amounts of salt.

The method of cooking and subsequent treatment of the cooked beans and cooking water were the same as in the previous experiments. An estimation was made of the quantity of salt left in the cooking water at the end of the experiment, which was then deducted from the total residue.

The average results obtained when unsoaked beans were cooked in tap water containing salt are given below:

Table VI.

Beans (100 *g.*) *cooked in tap water containing salt.*

(Average results.)

Parts of salt per 100 parts of water	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking
0·25	2 h. 0 m.	215	9·2
0·5	2 0	212	8·7
1·0	2 15	216	8·3

Discussion of results of Table VI.

These results show that contrary to the usually accepted statement, the addition of salt, in small quantities, to the cooking water, does not appreciably increase the time of cooking.

The loss in solid matter tends to decrease with the addition of salt (compare Table I), and the flavour is considerably improved.

The most satisfactory result was obtained when 0·25 % was added, the flavour of common salt being considered rather too pronounced when larger quantities were used.

In order to see how far these results were modified by the addition of salt when the beans were nearly cooked, some experiments were made in which beans were heated in boiling water for 15 minutes less than the average time required for cooking (*i.e.* 1 hr. 45 mins.), and 0·25 % of salt was then added and the heating continued until the beans were cooked. The results obtained confirmed those given in Table VI, since there was no appreciable difference in the times of cooking, whereas the loss in solids was on the average rather greater, being in fact practically the same as that observed in the case of untreated tap water.

The use of sodium bicarbonate and salt.

The results given in Sections II and III above show that the addition of sodium bicarbonate to the cooking water or preferably to the soaking water tends to decrease the time of cooking, but that the beans so cooked are somewhat insipid in flavour. The addition of salt, on the other hand, does not increase the time of cooking, improves the flavour and tends to reduce the loss of solid matter in cooking.

It therefore seemed probable that a judicious combination of the two methods would yield more satisfactory results than could be obtained by either method alone.

For this reason most of the experiments described in Section II (*A* and *B*) were repeated, the only variation made being the addition of 0·25 % of salt to the cooking water.

These experiments gave the anticipated results, and it is therefore hardly necessary to deal with them in detail.

The results as compared with those in Tables III–VI showed that the time of cooking was unaltered, the loss in solid matter on the average was slightly less and the flavour was improved.

IV. Cooking of dried legumes by steaming.

Cooking by the action of steam instead of by boiling water is a method frequently adopted on the large scale. This method has much to recommend it from the practical point of view, but its greatest advantage lies in the fact that the loss in solid matter during cooking must necessarily be less than when the food is exposed to the action of boiling water.

The possibility of cooking dried legumes by steaming was therefore investigated.

In the first experiments made, dry beans, and also beans which had previously been soaked in water, were placed in a steamer, closed with a tightly fitting lid. This method proved to be unsatisfactory both with dry and soaked beans, as even after steaming for about four hours the beans did not swell properly and presented a dried and unappetising appearance.

It appeared, therefore, that satisfactory results could probably only be obtained when the beans were covered with water during the process of cooking. The method was therefore modified, and a series of experiments was made in which the beans were covered with boiling water and then exposed to the action of steam. The method adopted was as follows:

Method of steaming beans. 100 g. of beans were placed in a basin covered with 250 cc. of boiling water, and the basin then placed quickly in a steamer, and closed with a tightly fitting lid. The cooked beans were separated from the water by straining and weighed.

Table VII.

Beans cooked by steaming.

Beans (100 *g.*) *covered with boiling tap water and cooked by steaming.*

(Average results.)

Treatment of beans	Time of cooking	Weight of cooked beans	Percentage loss of solids in cooking	Percentage loss of solids in soaking	Total percentage loss
I. Beans cooked without previous soaking	2 h. 10 m.	215	8·9	—	8·9
II. Beans cooked, without previous soaking, in water containing 0·25 % salt	2 10	217	8·4	—	8·4
III. Beans cooked, without previous soaking, in water containing 0·1 % sodium bicarbonate and 0·25 % salt	1 40	220	7·8	—	7·8
IV. Beans soaked overnight in tap water	2 5	214	8·6	1·8	10·4
V. Beans soaked overnight in water containing 1 % sodium bicarbonate and cooked in water containing 0·25 % salt	1 10	215	6·6	1·5	8·1

The water in the basin was evaporated to dryness and weighed, any necessary corrections being made as in the previous experiments.

The water below the steamer was evaporated to dryness and when allowance had been made for the solids present in the tap water, this residue was found to contain about 0·1 % of solid matter from the beans.

The results obtained in these experiments were such that it appeared worth while to pursue the matter further. In order to obtain a comparison with the results given in Tables III–VI, the experiments were repeated, using bicarbonate and also salt in the proportions previously found to be the most satisfactory. A summary of the results obtained is given in Table VII.

Discussion of results of Table VII.

These results show that although the beans were in contact with water during cooking, the loss in solid matter is less than when the beans are cooked in boiling water.

This is probably due to the fact that the water is not actually in a state of ebullition, and therefore the beans tend to break up less during the cooking process. In this connection it should be noted that the loss in solid matter observed in the individual experiments showed much less variation than in the case of boiling water (Table I). The actual values obtained in experiments (II) and (V) were as follows:

Percentage loss of solids in cooking.

	(II)	(V)
	8·5	6·6
	8·0	6·7
	8·6	6·4
Average	8·4	6·6

The time required for cooking is only very slightly greater than when boiling water is used.

The appearance and taste of the beans were considered to be superior to those cooked in boiling water, whilst as regards the action of sodium bicarbonate and of salt the results confirm the observations already made in the case of boiling water.

V. Note on haricot beans and dried peas.

Haricot beans. In order to compare the behaviour of other varieties of beans with that of butter beans, some of the experiments described in the foregoing sections were repeated using ordinary white haricot beans, and also

some brown beans which appeared to be fairly similar to the haricot beans but darker in colour.

The results were very similar to those obtained with butter beans. The time required for cooking was, however, slightly greater, being about 2 hrs. 15 mins.–2 hrs. 30 mins. when boiling water was used. The loss in solid matter was slightly less, namely 6–8 % of the total solids present in the beans.

Both these variations may be due to the fact that the haricot beans (white and brown) appear to have rather a thicker skin than butter beans.

Dried peas. Before discussing the behaviour of dried peas it is necessary to point out that the varieties of dried peas sold differ considerably in character. The peas sold in packets are usually smaller, have a better colour, and cook more rapidly than those sold by the pound. It is therefore difficult to make any general statement as regards the time required for cooking, and the loss in solid matter during cooking, though the latter shows an average from 6–8 % of the total solids in the peas. The experiments made show that soaking previous to cooking has a more marked effect in the case of peas than in the case of beans.

If the peas are cooked in tap water without previous soaking, the time required is, in most cases, greater than that required in the case of butter beans, the average time required with boiling water being about 2 hrs. 30 mins. Experiments carried out using the same sample of peas throughout, show, however, that the time of cooking can be reduced by about half-an-hour if the peas are soaked overnight in tap water previous to cooking.

The effect is still more marked if the peas are soaked in water containing sodium bicarbonate, since peas which required cooking for 2 hrs. 30 mins., if not previously soaked, cooked in 30 mins. after having been soaked overnight in tap water containing 1 % of sodium bicarbonate.

It is interesting to note in this connection that the effect of adding sodium bicarbonate to the soaking water appears to be much more generally recognised in the case of peas than in the case of beans, peas sold in packets sometimes containing a soaking powder or tablet. Those examined were found to consist chiefly of sodium bicarbonate, though the quantity supplied was usually rather in excess of that required. The use of sodium bicarbonate also tends to improve the colour of the peas, and it seems probable, from some experiments made with green vegetables which need not be dealt with here, that the colour is less affected by the cooking process if the peas are cooked in an open vessel, *i.e.* in a saucepan without the lid[1].

[1] This, however, increases the time required for cooking.

As regards the action of salt and the method of cooking described in Section IV the results obtained were similar to those observed in the case of the butter beans.

VI. Suggested methods of cooking dried legumes.

In carrying out this investigation it has been assumed that the dried legumes are to be served in the whole condition. Before considering how the results obtained in the foregoing experiments can be applied to the cooking of dried legumes on a large scale, some mention should be made of other possible methods of cooking.

It is clear that, if so desired, the cooking may be prolonged until complete disintegration takes place, and the beans, together with the cooking water, may then be served as a mash. In this case there can be practically no loss in solid matter during cooking. This method, however, is not satisfactory unless the skins are removed by some process, such as passing through a sieve. The removal of the skins entails a loss in solid matter of about 6·5 %, but to what extent this loss represents a loss of food material is a matter which lies outside the scope of this enquiry.

Another method which appears to be specially favoured in the United States [Wait, 1907] is to parboil the beans in water containing sodium bicarbonate, then to remove the skins by rubbing and finally to bake the beans in an oven with the addition of fat.

This method, apart from the question of the doubtful advisability of removing the skins appears to involve too many processes to be satisfactory on a large scale, and has therefore not been dealt with in detail.

From a consideration of the results obtained in Sections I–V the following methods are suggested for cooking dried legumes on a large scale.

Method I. Soak the beans overnight or for a period of not less than four hours in water containing 1 % of sodium bicarbonate (1½–2 oz. sodium bicarbonate per gallon of water)[1] using about half a gallon of water to 2 lb. of beans. Strain off the soaking water and cook by one of the following methods:

(*A*) Place the beans in a dish or basin and cover with boiling water (about ½ gallon to 2 lb. beans) containing 0·25 % of salt (¼ to ½ oz. salt per gallon)[2] and cook in a steamer.

The time required for cooking by this process is about 1¼ hours.

[1] The exact figure is 1·6 oz. per gallon, but the above directions are probably sufficiently accurate for working on a large scale in the kitchen. Quantities much in excess of this amount should not be added (see Section II *B*).

[2] The exact figure is 0·4 oz. salt per gallon; quantities much in excess of this amount should not be added (see Section IV).

(*B*) Place the beans in a saucepan, add tap water (1 gallon to 2 lb. of beans) containing 0·25 % of salt, and cover with a lid and cook by bringing the water to the boil.

The time required for cooking by this process is about 1 hour.

Where possible, method (*A*) should be employed since it is more economical as regards loss of solid matter in cooking besides giving more satisfactory results in other respects. Under some circumstances soaking previous to cooking may prove to be inconvenient; in such cases the beans may be cooked without previous soaking, but a longer time is then required for cooking.

Attention must, however, again be drawn to the fact that unless sodium bicarbonate is added to the soaking water there is little to be gained (except as regards colour—see Section I) by soaking the beans previous to cooking. If it is inconvenient to soak the beans previous to cooking, Method II described below may be used, but it is not satisfactory in the case of peas (see Section IV), and it should be understood that even in the case of beans Method I (*A* or *B*) is distinctly preferable.

Method II. (*A*) Place the beans in a basin or dish, cover with boiling tap water ($\frac{1}{2}$ gallon to 2 lb. beans) containing 0·1 % of sodium bicarbonate ($\frac{1}{4}$–$\frac{1}{2}$ oz. per 2 gallons of water)[1] and 0·25 % of salt and cook in steamer.

The time required for cooking by this process is about 2 hours.

(*B*) Place in a saucepan, add tap water (1 gallon to 2 lb. beans) containing salt and sodium bicarbonate in the proportions given in (*A*), cover with a lid and cook by bringing the water to the boil.

The time required for cooking by this process is about $1\frac{3}{4}$ hours.

For the reasons stated above (*A*) should be used where possible in preference to (*B*).

The directions given above are based on experiments which have necessarily been made on a small scale, and it is possible that in carrying out the processes on a large scale it will be found necessary to introduce certain modifications.

The quantity of sodium bicarbonate used in Method II will, as already pointed out, need adjusting according to the type of water used for cooking.

On a large scale the proportion of water to beans and the time of cooking may also work out rather differently, and the above directions should be looked upon as a general guide, which by means of a few experiments made in the kitchen can be modified in accordance with the scale required and the equipment available.

[1] The exact quantity is 0·16 oz. per gallon; quantities much in excess of this amount should not be added (see Section III *A*).

In conclusion it is desired to emphasise the fact that in carrying out this investigation primary importance has been attached to the practical aspects of the question, and it is realised that there are several factors which need to be dealt with more fully. In particular information is needed as to the composition of the residues obtained from the different cooking waters, and the nature of the action of sodium bicarbonate and salt on the dried legumes, and it is hoped to make such work the subject of further enquiry.

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XXI. SOME OBSERVATIONS ON YEAST GROWTH.

By ARTHUR SLATOR.

(*Received June 8th, 1918.*)

It has been shown for many growths of micro-organisms that the logarithmic law of increase holds good so long as growth takes place unrestrictedly in the nutrient medium. If N is the number per cc. at time t then $\frac{dN}{dt}\Big/N$ is constant during this period of growth. This value $\frac{dN}{dt}\Big/N$, which we may call Z, is the rate of growth per cell or the constant of growth and we have the equation $Z = a$, a constant[1].

The generation-time (G.T.) $= \frac{\log 2}{Z}$.

When an old culture of bacteria or yeast is seeded into a fresh nutrient medium the initial development takes place slowly. During this lag-phase in growth Z is initially small, it increases with the time and finally reaches the value of unrestricted growth.

It has been shown by Ledingham and Penfold [1914] that the growth of *B. coli* during this period of lag follows a definite law. On calculating Z from their equation[2] we find

$$Z = at^b,$$

where a and b are constants in each experiment but vary in the different experiments with different seedings. In eight experiments b has the values 0·88, 0·77, 0·56, 0·56, 0·97, 0·74, 1·01, 1·7. It has been further pointed out

[1] This logarithmic constant occurs in many branches of science and there are many ways of expressing it. The "constant" of a unimolecular chemical reaction, the period of "half-change" of a radioactive substance, the "birth rate" of a population, the "generation-time" in the growth of micro-organisms, "compound interest" on a sum of money, are some of the many examples which can be quoted. For a general method of recording such "velocity-constants" the inverse of the "generation-time" or the inverse of the period of "half-change" has much to recommend it and is in some respects simpler than that suggested by J. W. McBain [1917]. In this paper the usual method of giving velocity-constants is employed, but generation-times are also given.

[2] Ledingham and Penfold's equation reads $X^n = k \log Y$ where X is the time, and Y the number of bacilli (seeding = 1), k and n being constants. If the equation is put in the form $X^n K^n = \log Y^n$, K the new constant keeps the same value for the whole series of experiments.

Differentiating the equation we get

$$\frac{dY}{dX}\Big/Y = \frac{K^n X^{n-1}}{0{\cdot}434} = \frac{K}{0{\cdot}434}(KX)^{n-1}.$$

Substituting Z for $\frac{dY}{dX}\Big/Y$, a for $\frac{K}{0{\cdot}434}$, t for KX, and b for $n-1$, we have $Z = at^b$.

[Slator, 1917] that this equation can be slightly simplified. If a suitable unit of time be chosen the constant a in the equation $Z = at^b$ remains constant for the whole series of experiments. This equation $Z = at^b$ is not usual in chemical dynamics and it is of some interest to know whether it has any biological significance. Some interesting facts relating to growth of plants and animals are discussed by D'Arcy W. Thompson [1917, Chapter III]. The regenerative growth of tadpoles' tails seems to follow a law similar to that given above. Examples of rhythmic growth are also given.

It was in the hope of finding another example of this law that the lag-phase in the growth of yeast was investigated. It was found however that this period of growth is short and does not admit of measurements such as Ledingham and Penfold have carried out. The results are described below. An account of some further experiments on yeast growth is also included.

Method of measuring the rate of growth.

Direct observation of the growth of yeast cells under the microscope was found to be most suitable for the purpose of measuring the lag-phase of growth. A hollow slide was constructed through which water at any desired temperature could be circulated. By means of such a "warm slide" the medium in which the yeast cells were growing was kept at a desired constant temperature and a number of yeast cells were continually under observation under the microscope. Other methods of keeping the slide at a constant temperature would no doubt be effective. It is evidently of some convenience to deal with a rapidly growing yeast and most of the experiments were carried out with a baker's yeast growing at 30° in malt-wort of sp. gr. 1·040.

Baker's yeast growing unrestrictedly at 30°.

Such growth was observed to take place in the following manner. A bud appears and grows steadily for about $\frac{3}{4}$ hr when it has developed to about the same size as the mother cell. For another $\frac{1}{2}$ hr no appreciable change is to be noticed. Both cells then bud usually almost simultaneously and the process of growth is then repeated. By counting the cells at various intervals of time fair constants of growth can be obtained. In the following table t = time in hours, $N + n$ = the number of cells at time t, the initial number being N. K is calculated according to the equation

$$0{\cdot}434\, K = \frac{1}{t} \log \frac{N+n}{N}.$$

Experiments 1 and 2 are single colony growths, in Exps. 3 and 4 groups of four colonies were counted.

TABLE I.

1	t	0	$1\frac{1}{2}$	$2\frac{1}{2}$	4	
	$N+n$	$4\frac{1}{4}$	10	16	35	
	$0{\cdot}434K$	—	0·248	0·230	0·229	Average = 0·236
2		Repeat exp. gave				„ = 0·244
3	t	0	1	$1\frac{1}{2}$	3	
	$N+n$	$9\frac{3}{4}$	17	$28\frac{1}{4}$	57	
	$0{\cdot}434K$	—	0·241	0·264	0·256	„ = 0·254
4		Repeat exp. gave				„ = 0·248

Average of the four = 0·246, G.T. = 1·22 hrs = 73 mins.

Another method of measuring this generation-time gave good results. It is carried out in the following manner. A yeast cell is observed to bud and the time t_1 noted. The bud grows and eventually two new buds appear, one on each cell usually within a few minutes of each other (Fig. 1). The mean time t_2 is observed. The difference t_2-t_1 is the time of a complete generation. The method is suitable for estimating the G.T. of individual cells. Twelve cells watched in this way under the microscope gave values 78, 66, 76, 70, 79, 65, 81, 72, 72, 70, 72, 73 the average being 73 mins. The G.T.s of individual cells vary therefore up to 8 mins on each side of the average.

TABLE II.

Cell number	Time of appearance hrs mins	Average hrs mins	Time when fully grown hrs mins
2	0 0	0 0	0 45
3	1 18		
4	1 16	1 17	2 0
5	2 33		
6	2 33		
7	2 33		
8	2 33	2 33	3 15
9	3 49		
10	3 50		
11	3 52		
12	3 38		
13	3 45		
14	3 48		
15	3 52		
16	3 38	3 47	

Generation times = 77, 76, 74, average 76 mins.

The following case of a single cell growing to sixteen cells is of interest. The time at which each cell appeared as a bud was noted and each successive average G.T. obtained from the time differences. In the table the number of each cell corresponds to that given in the diagram (Fig. 2).

The lag-phase.

If an old culture of yeast is seeded into wort an hour to two hours elapses before visible growth takes place. It is then found that growth proceeds at the maximum rate.

A five days old culture of yeast was seeded into fresh wort and the G.T. of thirteen cells estimated as soon as buds appeared. They gave values 81, 89, 80, 78, 77, 72, 85, 74, 88, 78, 76, 81, 70 mins; average 81 mins, a value only slightly greater than the minimum G.T. When older cultures were used similar results were obtained but more dead cells were present.

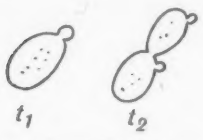

Fig. 1. Generation-time $= t_2 - t_1$.

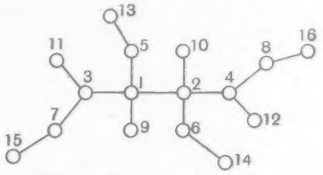

Fig. 2. Diagram of yeast growth.

Individual cells show considerable variation in the time they remain quiescent before budding. Thirty-seven cells kept under observation gave the following results:

During the first 60 mins.		0 budded	from 91 to	95 mins.	2 budded
from 60 to	65	6	96	100	4
66	70	7	101	105	0
71	75	4	106	110	1
76	80	8	111	115	1
81	85	1	116	120	1
86	90	1	121	125	1

The lag-phase in the growth of a yeast cell consists therefore of a quiescent stage of one or two hours varying with the individual cells and then growth at the normal unrestricted rate takes place.

Yeast growth proceeds in alternate periods of growth and rest and the lag is apparently little more than a prolonged period of rest. Many attempts have been made to deduce the equation $Z = at^b$ from such data but without success.

If it were possible to follow the growth of *B. coli* under the microscope and compare it with the results obtained by plating, an explanation of Ledingham and Penfold's equation might be forthcoming. It is worth noting that the death of some of the cells may take place during such measurements. Thus Penfold [1914, Table X, p. 230] records an initial decrease in the number of bacilli in some cases; that is a certain number of the original seeding die in the nutrient medium but grow if gelatin is present. The presence of a poison which diffuses slowly might account for the favouring influence of gelatin.

The growth of spores.

It was thought possible that the development of yeast spores might give a longer period of lag than is the case with quiescent yeast cells. Experiments were therefore made with a wild yeast which spores abundantly. Measurements of the G.T. of the yeast growing in malt-wort at 30° gave values 91, 82, 84, 81, 92, 95 mins, average 88 mins. When spores are kept under observation on the warm slide the general characteristics of spore growth are observed; the long period of rest (often 24 hrs) before visible development, the bursting of the cell walls and the budding of the spores. This wild yeast developed at first small round cells which grew in a mass and could not be counted. After about the second generation the normal larger cells developed, the rate of growth of which was easily estimated. Three cells gave values 94, 85, 88 mins, that is the maximum rate of growth is already attained. There is therefore little or no lag when once the spores have started developing.

Influence of carbon dioxide.

To obtain the maximum rate of yeast growth (minimum G.T.) it is essential that there should be no appreciable accumulation of carbon dioxide in the nutrient medium. This is the case if the number of yeast cells does not exceed a few hundred thousand per cc. That carbon dioxide has an appreciable retarding influence is easily demonstrated. If the experiments are carried out with wort saturated with carbon dioxide as the nutrient medium, a number of the cells are observed to die and those which survive grow at about half the maximum rate. Yeast which has been growing some time in the presence of carbon dioxide no longer exhibits the dying off effect and grows rather faster, but in no case was the maximum rate of growth observed. The effect of carbon dioxide on yeast growth is well known and seems to be general. Air which has a large influence in the later stages of yeast growth has no influence when dealing with these small concentrations of yeast, for rates of growth measured under these conditions in wort saturated with air and in wort from which the air had been evacuated gave the same result.

Yeast growth at high temperatures.

If rates of growth at different temperatures are plotted against the temperature a characteristic curve is obtained. Below a certain low temperature and above a certain high one no growth takes place. Between the two the rate of growth varies greatly. As the temperature rises the rate increases and finally attains a maximum. A further increase in the temperature causes a rapid

decrease in the rate which finally becomes zero. In an investigation of the influence of temperature on the rate of growth of *B. delbrücki* it was observed that the rate was about halved when the temperature was raised from 50° to 52·5° [Slator, 1916]. The rate however remained constant at the higher temperature. It is of interest to know whether the lower rate is due to cell destruction or to slower rates of growth of the individual cells. By observing growth on the "warm slide" this point can be settled as far as yeast cells are concerned. The baker's yeast used in these experiments gave an average G.T. of 63 mins at 39° to 40°. At 41° retardation in growth takes place and some of the cells also die. Observations of a group of four cells growing at 41° gave the following result, one cell died before budding and the other three gave G.T. 67, 77, 85 mins. Other groups of cells showed similar results—some dying and some growing. At these high temperatures therefore some of the cells grow at about the maximum rate, some at a considerably lower rate and some die.

The rate of spore production in yeasts is influenced by temperature in a similar manner to growth. These rates are usually measured in the following way. An actively growing culture is spread over a moist gypsum block and kept at a constant temperature. An examination is made periodically and the time of the first appearance of spores is noted. The times observed are therefore the minimum for spore production at the given temperature. If one constructs curves plotting temperature against the inverse of these times the similarity to temperature-rate of growth curves is very noticeable. Here again we have the upper and lower limiting temperatures, a maximum rate, and a part of the curve where the temperature coefficient is less than one. We are dealing here only with the few cells which form spores in the shortest possible time and the slower rates at high temperature cannot be explained by cell destruction. Probably both with spore production and with growth at temperatures above that of maximum rate partial destruction of some substance affecting the rate takes place but is not sufficient to stop development entirely.

In the case of enzyme action where a similar maximum occurs temperature coefficients less than one are usually attributed entirely to destruction of the enzyme.

Other uses of the "warm slide."

This method of investigating yeast growth can be conveniently used in all cases when rapid growth takes place. It is also of use in determining the initial effect of poisons on growth, thus a dying off effect or a lag-phase is soon detected by this means whilst they may be missed entirely if rates of growth are estimated by ordinary counting methods.

Use of the "warm slide" was made to throw some light on the effect of yeast growth on diastatic action. The experiments showed no new results but they suggested a point which is worth mentioning. Diastase (malt extract) has a slow solvent action on malt starch at ordinary temperatures and this reaction is accelerated if yeast is present. G. H. Morris [1901] showed that the products of reaction of diastase on starch have no influence on the rate of the reaction and it is unlikely therefore that removal of the reaction products by fermentation would account for the acceleration. The action of diastase on starch is accelerated by carbon dioxide [for references see Euler, 1912, p. 158] and no doubt the carbon dioxide evolved by the yeast influences the reaction. Whether this effect accounts for the acceleration entirely requires other experiments to prove but it certainly has to be taken into consideration.

The influence of oxygen on yeast growth.

The action of air (oxygen) on yeast growth has attracted attention from the time of Pasteur onwards and rightly so, for oxygen plays an important part in yeast growth. One of the most striking ways of showing that air is necessary to yeast growth is to attempt to grow "giant colonies" of yeast on wort gelatin in an evacuated vessel. Growth is very poor and liquefaction of the gelatin soon takes place under these anaerobic conditions.

Within the last few years H. T. Brown [1909, 1914] has more thoroughly investigated this influence of oxygen. In agreement with A. J. Brown [1905] he shows that when yeast develops in malt-wort, growth is retarded and finally ceases at an earlier stage than one would expect taking into consideration the food requirements of the yeast and the retarding influence of alcohol and carbon dioxide on growth. He concludes that lack of oxygen is the main factor which causes this retardation. The experiments described here are in accordance with this conclusion. He further finds that very considerable yeast growth takes place under strictly anaerobic conditions. This admits of explanation on the assumption that there is a certain amount of chemically combined oxygen in the wort which is available for yeast growth. Subsequently [1914] H. T. Brown discards this explanation and considers that the yeast used for seeding carries oxygen with it in the form of an "oxygen charge" and that growth is determined by this "charge."

The theory gives a ready explanation of the approximately rectilinear curves of growth to which he draws attention. The alternative explanations depend on whether retardation in growth is due to change in the medium (lack

of combined oxygen) or to change in condition of the yeast (continual splitting of the "oxygen charge").

TABLE III.

Aerobic growth at 20°			*Anaerobic growth at* 20°		
t (hrs)	$N+n$	$0{\cdot}434K$	t	$N+n$	$0{\cdot}434K$
0	11,500	—	0	11,500	—
22·5	630,000	0·077	42 hrs	322,000	0·034
42	11,200,000	0·071			
			0	226	—
0	226	—	4¾ days	266,000	0·027
42½	175,000	0·068	5 „	1,440,000	0·032
47	441,000	0·070			
			0	4·4	—
0	4·4	—	9 „	7,000,000	0·029
66½	365,000	0·074			0·031
		0·072			
G.T. = 4·2 hrs			G.T. = 9·7 hrs		

Estimations of the rate of growth with very small seedings were made to decide between these two alternatives. The experiments described below show fairly conclusively that there is in malt-wort combined oxygen available for yeast growth. Rates of growth were estimated by yeast counts according to Method 1 previously described [Slator, 1913], a Burton brewery yeast being used for the purpose. It grew at a rate corresponding to a G.T. of 3 hrs at 20° in malt-wort of sp. gr. 1·040, and the same results were obtained whether the experiments were carried out in the presence of air or in evacuated tubes. All attempts to obtain lower rates by drastically excluding air were unsuccessful. Efforts were then made to find a medium in which growth is influenced by air. Half-fermented wort in which yeast growth had stopped was filtered and partly evaporated in an open dish to free it from alcohol and growth in this medium tested. It was found however that yeast grew in this medium both anaerobically and aerobically at the same rate. Wort of sp. gr. 1·060 was then taken, fermented until it contained about 2 % of alcohol and filtered out of contact with air. It was then evaporated in an evacuated distilling flask to bring it back to about its original gravity of 1·060. In this medium yeast behaved differently. Rates of growth under anaerobic conditions were much smaller than under aerobic conditions. Table III gives the results of measurements of rates of growth with different seedings; in one case the experiments were carried out with free access of air, in the other under anaerobic conditions in a number of evacuated tubes. A G.T. of 4·2 hrs was observed when air was present and a G.T. of 9·7 hrs when air was absent. The seeding in each case

was made with yeast which was grown under anaerobic conditions. The presence of air was found to favour the growth of smaller cells than were produced when air was absent, but as the increase over the period of growth is great the difference in the size of the cells would hardly affect the velocity-constants.

There is no doubt that partly fermented wort differs from the original wort in that combined oxygen available for yeast growth is present in the one case and very much reduced in the other. The effect of oxygen on yeast growth deserves more attention than is given in this short note, but apparently dissolved oxygen (which is rapidly absorbed by the yeast) and oxygen chemically combined in the wort are both available for yeast growth. The necessary amounts are small; thus Brown [1914] estimates that 1·7 cc. of oxygen are used up in the growth of 10^{10} cells (about 2·5 g. of pressed yeast).

Summary and discussion of results.

A method of measuring rates of growth of yeast by direct observation under the microscope is described. The G.T. of individual cells of a baker's yeast growing in malt-wort at 30° varies up to 10 % on each side of the average value of 73 mins.

Measurements of the lag-phase in the growth of yeast cells show that old yeast cells remain quiescent for some time when introduced into a fresh medium, the time of quiescence varying with different cells. They then start growing at the normal unrestricted rate. Yeast growth proceeds in alternate periods of rest and growth, and lag is apparently little more than a prolonged period of rest.

Little or no lag is observed in yeast cells grown from spores, when once development has started.

The retarding influence of carbon dioxide is readily observed by this method of measuring growth.

At a temperature above that of maximum rate of growth some cells grow at about the maximum rate, some at lower rates and some die.

The influence of oxygen on yeast growth has been tested by measuring generation-times under anaerobic and aerobic conditions. The results confirm previous conclusions that air is necessary for yeast growth, and that oxygen chemically combined in the wort as well as dissolved oxygen which is rapidly absorbed by the yeast is available for yeast growth. A method of preparing a medium in which yeast growth is sensitive to air is described.

If yeast is seeded into malt-wort the following stages of growth may be observed: the lag-phase or period of quiescence, the logarithmic phase or period of unrestricted growth, retardation in growth due to carbon dioxide, retardation in growth due to failure of oxygen. The latter factor is the main one which finally stops growth. The accumulation of alcohol and lack of necessary nutrients seldom come into consideration up to this stage. When fermentation is over air may have access and cause a regrowth of the yeast, the alcohol and possibly lack of nutrients such as assimilable sugar are then the main retarding factors.

It depends on conditions of seeding, aeration, temperature, etc., how far these different stages of growth become prominent, overlap or disappear. Thus if the seeding consists of actively growing yeast the lag-phase disappears, if the seeding amounts to a few million per cc. retardation starts at once and there is no logarithmic period, if the seeding is very great lag in growth and rapid accumulation of retarding influences may prevent growth entirely. The problem of the final yeast crop is largely a matter of rates of growth with failing supplies especially of oxygen.

Thanks to the work of A. J. Brown, A. L. Stern [1899, 1901], H. T. Brown and others it should not be difficult to determine rates of growth with definite small concentrations of known essential nutrients and possibly put the problem of yeast crops on a sound mathematical basis.

In this and in previous papers [1913, 1916] several methods of measuring the logarithmic constant of growth and the generation-time of micro-organisms have been described, and attempts have been made to show the importance of these measurements in an investigation of the growth of micro-organisms and the chemical action brought about by them. The figures obtained are not merely interesting mathematical curiosities. They represent accurate measurements of growth under conditions where *the medium remains practically unaltered*, and they are of considerable value in solving problems connected with the growth of micro-organisms. It may be said that yeast (for instance) for practical purposes never grows under such conditions and that measurements under "normal" conditions are of more value. The argument is only partly correct for one can investigate phases of retarded growth (such as when yeast growth is retarded by lack of oxygen) by this method, and in general it is better to study each part of the puzzle under the simplest possible conditions and then attempt to fit the pieces together into a harmonious whole. The method is in fact that of investigating growths of micro-organisms and the chemical actions brought about by them in the same manner as one would investigate the

kinetics of a purely chemical reaction. One has every confidence that the method will justify itself in throwing fresh light on these problems. Much of the work carried out in connection with the fermentation industries especially relating to growths of yeast, acetic acid bacteria, lactic acid bacteria, etc., is sufficiently far advanced to be investigated from this point of view and such measurements would be well worth making. The field of investigation is by no means limited to these organisms but extends to all branches of science in which micro-organisms play a part.

In conclusion I desire to express my thanks to Dr A. L. Stern, with whom I have had the advantage of discussing the subject matter of the present paper.

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XXII. THE ANTISCORBUTIC FACTOR IN LEMON JUICE.

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In a previous communication [1918] we have described an attempt to isolate the antiscorbutic principle by means of adsorption. The attempt proved unsuccessful but we were able to make a series of observations in connection with the behaviour of the antiscorbutic factor in orange juice towards some adsorbents under certain conditions. We have since attempted to study the chemical nature of the factor in question adopting another method of investigation, namely that of fractionating an antiscorbutically active juice. We have chosen lemon juice for that purpose. As is well known the main bulk of the solids of lemon juice consists of citric acid. In the event of the antiscorbutic being associated with the citric acid, good yields of the acid could be obtained from the juice for further investigation. If on the other hand by removing the citric acid the solid residue remained active, the antiscorbutic would then be associated with a comparatively much smaller mass of extraneous matter offering a better opportunity of studying both its chemical and physiological properties. The latter has proved to be the case and we now describe in this communication a few experiments from an investigation in progress on the above lines.

Experimental.

The following procedure was adopted in order to remove the free citric acid from lemon juice. To freshly squeezed out lemon juice an excess of precipitated calcium carbonate was added with constant stirring. The resulting suspension was then treated with two volumes of absolute alcohol and filtered through a fluted filter. The residue remaining on the filter was then pressed out in a hand press and the liquid thus obtained filtered. Both filtrates, which should be clear, were combined and evaporated in vacuo at 35° C. until all

the alcohol had been removed and were then made up with distilled water to the original volume of the lemon juice used. The solution thus obtained is very slightly acid to litmus, yellow in colour, sweetish to taste and retains the characteristic lemon flavour as well as the bitter taste of the lemon. On evaporating the solution to dryness and heating the residue at 110° for an hour the equivalent of 1 cc. of the original lemon juice yields a residue weighing about 15 mgms. The residue after heating becomes caramelised and syrupy and tastes very sweet. It reduces Fehling's solution freely. On incinerating the residue about 3 mgms. of inorganic constituent per cc. of original lemon juice is obtained. The inorganic residue resulting from 10 cc. lemon juice treated as above when dissolved in a known quantity of decinormal H_2SO_4 and titrated back required 2 cc. of $N/10$ H_2SO_4 to neutralise the carbonates produced by the incineration of the small quantities of the salts of organic acids present and this when calculated on the basis of sodium citrate corresponds to 1·7 mgms. per cc. When the decitrated juice was treated with $CaCl_2$ and two volumes of absolute alcohol a scanty precipitate of a few mgms. was obtained from 10 cc. of the solution and this consisted of the calcium salt of some organic acid or acids, as it charred and smelled of burning sugar when heated. A sample of lemon juice evaporated to dryness and heated at 110° for one hour gave a total residue of 105 mgms. per cc., 4 mgms. of which was inorganic.

Experiments were instituted to test the therapeutic and other properties of the treated lemon juice.

Expt. 1. The purpose of this experiment was to test the antiscorbutic potency of the treated lemon juice. Quantities equivalent to 2 cc., 3 cc., 5 cc. and 7 cc. were tried respectively on four guinea pigs. The animals received oats and bran *ad lib.* and 50 cc. of autoclaved full milk per diem. The respective doses of the treated lemon juice were administered daily by hand. The animals were allowed to go on for about eighty days when they were all chloroformed. It will be seen from fig. 1 which represents the weight curves of the animals, that on 2 cc. guinea pig No. 71 just managed to maintain its weight. Although it was unable to grow on that dose it failed nevertheless to succumb to scurvy. The other animals in the experiment actually succeeded in putting on weight. At the post mortem examination guinea pig No. 72, which received a dose of 3 cc. showed slight haemorrhages in the femoral muscles and also slightly enlarged costochondral junctions. The other animals (Nos. 54, 55 and 71) showed no signs of scurvy at all. This experiment shows without any doubt that after removing the free citric acid the residual fraction of lemon juice retains some antiscorbutic activity.

Expt. 2. Our next aim was to ascertain approximately what proportion of the antiscorbutic content of lemon juice remains in the residue after the removal of the acids. It has previously been pointed out by us in another communication [1918] that with our present technique it is impossible to carry out quantitative determinations of the antiscorbutic content of a solution with any great precision. It is nevertheless possible to ascertain roughly the relative antiscorbutic potency of two solutions by administering equal quantities of the solutions to guinea pigs of approximately the same weight kept on a scorbutic diet and watching the clinical developments. We have adopted this procedure in this experiment. Doses of 0·5 cc., 1 cc. and 1·5 cc.

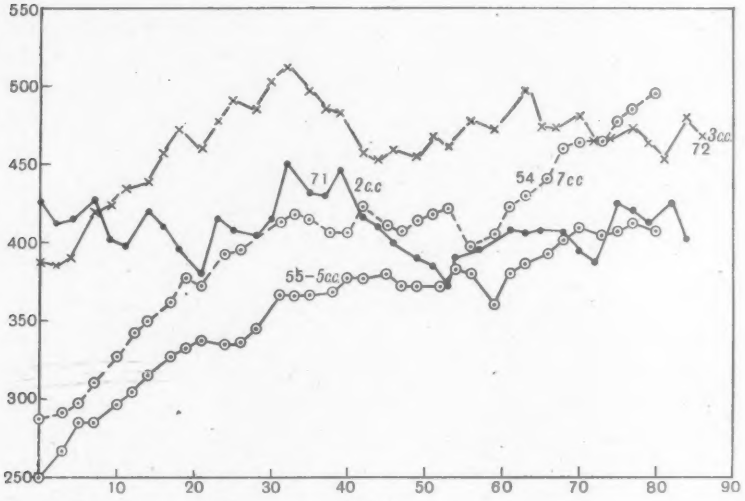

Fig. 1

each of treated and untreated lemon juice respectively were given to six guinea pigs. Animals Nos. 263, 267 and 268 received 0·5 cc., 1 cc. and 1·5 cc. of untreated lemon juice respectively, while Nos. 266, 264 and 265 received 0·5 cc., 1 cc. and 1·5 cc. of treated lemon juice. Fig. 2 represents their weight curves. Guinea pigs Nos. 263 and 266 receiving 0·5 cc. of untreated and treated lemon juice respectively died of scurvy within about a month, showing that 0·5 cc. either of treated or untreated lemon juice is insufficient to prevent guinea pigs from succumbing to scurvy. No marked differential clinical features could be observed in these animals. The behaviour of Nos. 264 and 267 which received a dose of 1 cc. was also very similar. They were both chloroformed

after forty-two days and at the post mortem they both disclosed signs of scurvy. The weight curve of No. 267 which received the untreated lemon juice is however a little better than that of No. 264 which was dosed with the treated juice. Guinea pigs Nos. 265 and 268 received 1·5 cc. of treated and untreated lemon juice respectively. Neither of these animals developed scurvy. Unfortunately No. 268 succumbed after thirty-seven days to an intestinal complaint, which is often the case with guinea pigs receiving high doses of lemon juice. Not the slightest trace of scurvy could however be found at the post mortem examination. No. 265 was chloroformed after forty-two days and was found to be in excellent condition. Nothing abnormal was disclosed by the post mortem examination. It is seen then that the three sets of pigs behaved almost identically, showing that the relative antiscorbutic potency of the treated and untreated lemon juice is practically the same, from which one may safely conclude that little if any of the antiscorbutic is associated with the free acids in the lemon juice of the solids of which they form the main portion.

Expt. 3. We endeavoured next to ascertain whether treated lemon juice is antiscorbutically stable for short periods of time. Small portions of treated lemon juice were stored in the cold room in corked flasks for about a fortnight before being tested. Quantities of 3 cc., 5 cc. and 8 cc. were tried on three guinea pigs. As will be seen from fig. 3 all the animals failed to succumb to scurvy within seventy-four days, after which period they were chloroformed. No. 76 which received 8 cc. and No. 74 which received 3 cc. of the treated extract showed very slight signs of scurvy at the post mortem examination. The other guinea pig was quite normal. As the weight curves show, none of the animals grew satisfactorily. The stored juice then seemed to have deteriorated to some extent during the fortnight's cold storage although it retained the best part of its antiscorbutic potency.

Expt. 4. It was of interest to see whether on evaporating the treated lemon juice the residue would retain its antiscorbutic power. We therefore evaporated a litre of the treated lemon juice at a temperature of 30°–40° C. in vacuo until the residue assumed a thick syrupy consistency. The residue was then transferred to an evacuated desiccator where it was kept during the experiment. Three pigs, Nos. 68, 69 and 70, received 100 mgms., 150 mgms. and 200 mgms. per diem of the residue respectively in addition to the scorbutic diet. The doses were dissolved in suitable quantities of distilled water just before administration. All the pigs died of scurvy within 42–50 days. Fig. 4 represents the weight curves of the above animals. It is seen that the doses given have

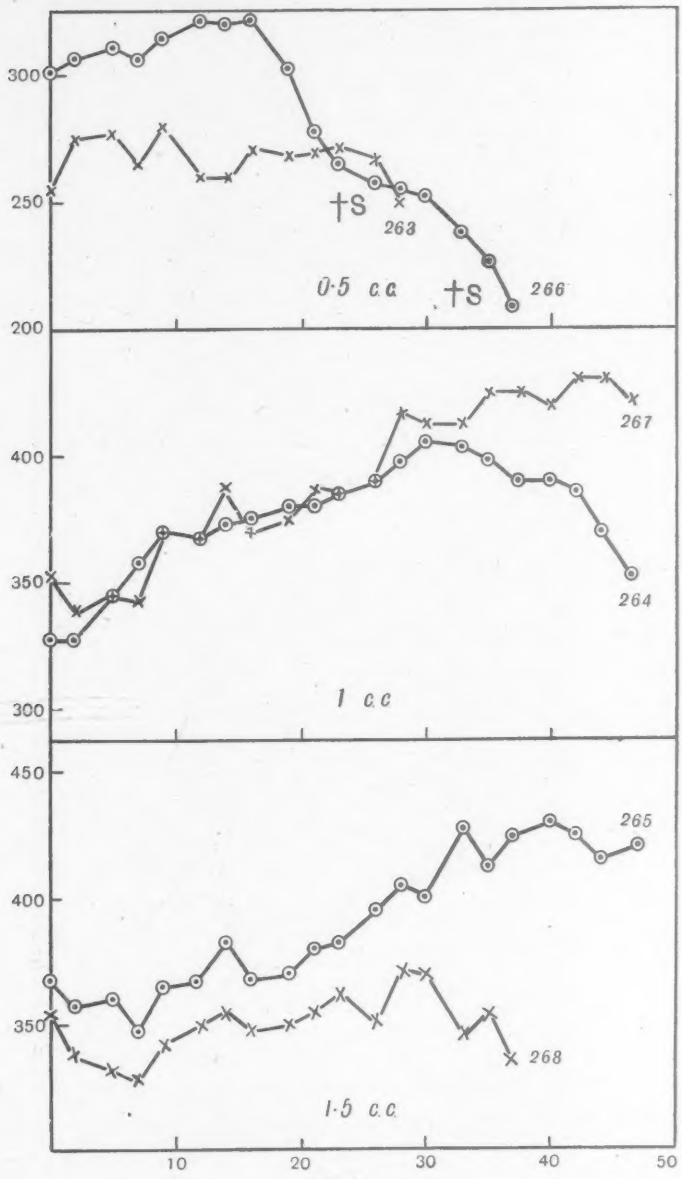
300
250
200
†S
263
0·5 c.c.
†S
266
400
350
300
267
264
1 c.c.
450
400
350
265
268
1·5 c.c.
10
20
30
40
50

Fig. 2

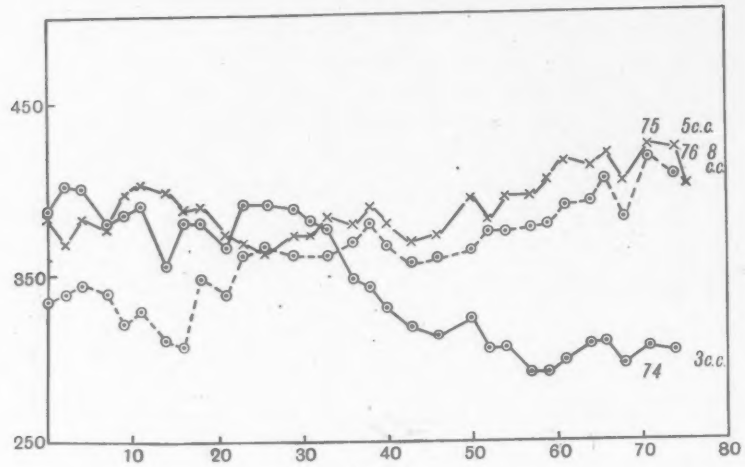
450
350
250
10
20
30
40
50
60
70
80
75 5c.c.
76 8 c.c.
74 3c.c.

Fig. 3

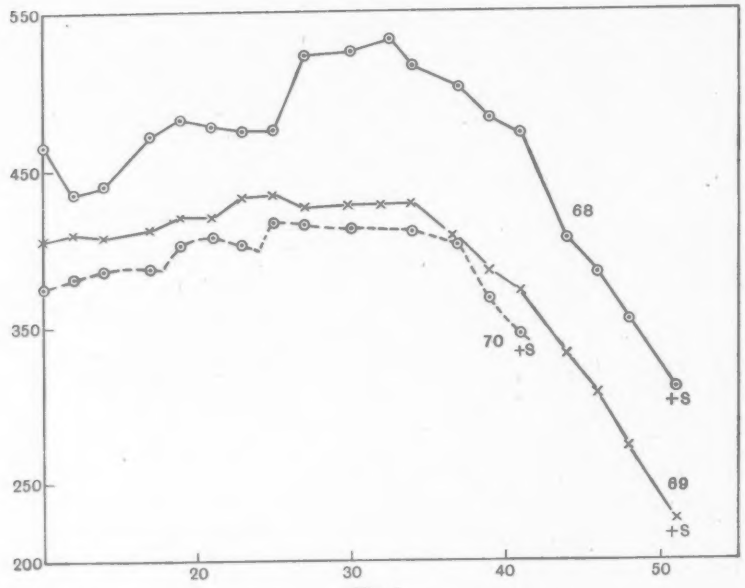
550
450
350
250
200
20
30
40
50
68
70 +S
+S
69
+S

Fig. 4

delayed slightly but not prevented the onset of scurvy. As the doses given were comparatively high it is plain that by drying the treated lemon juice in the way we have just described the best part of its antiscorbutic activity is destroyed.

Expt. 5. From experimental evidence at our disposal obtained in the course of another investigation we decided to modify the process of desiccation by previously acidifying the treated lemon juice. After the precipitation of the free acids of the lemon juice with $CaCO_3$ and alcohol 1 gm. of citric acid was dissolved in the alcoholic filtrate of 1 litre of lemon juice. It was then evaporated *in vacuo* at 30°–40° as before. A residue of 14 gms. was obtained.

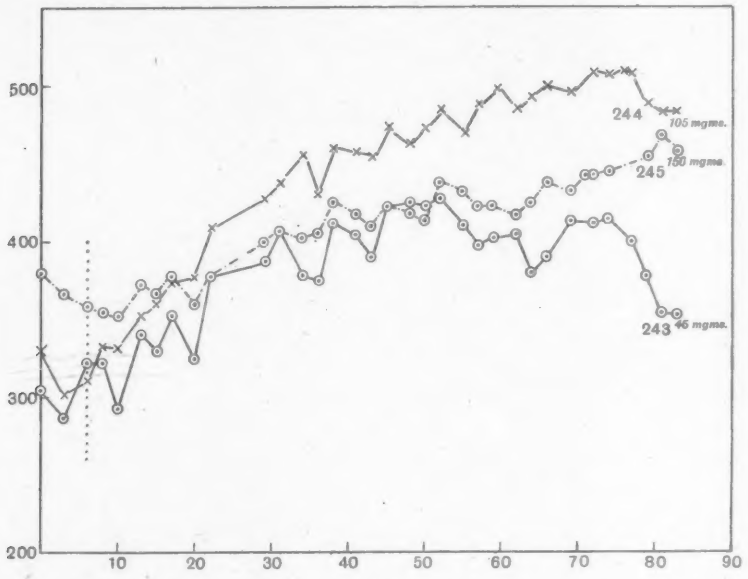

Fig. 5

Guinea pigs Nos. 243, 244 and 245 received 45 mgms., 105 mgms. and 150 mgms. respectively besides the usual scorbutic diet. As before the dry residue was dissolved before administration. As will be seen from fig. 5 which represents the weight curves of these animals they were all increasing in weight. After eighty-three days the animals were chloroformed and at the post mortem examination were found to be in excellent condition. Not the slightest signs of scurvy were to be observed. It is thus seen that by acidifying slightly the

medium, treated lemon juice can be evaporated to dryness yielding a residue of high antiscorbutic potency.

Expt. 6. When pigeons suffering from an acute attack of polyneuritis are treated with subcutaneous or intramuscular injections of highly potent anti-neuritic extracts a cure is brought about even with greater rapidity than when the treatment is carried out *per os*. Holst and Frölich [1912] administered cabbage juice intraperitoneally to guinea pigs subsisting on a scorbutic diet as preventive doses but were unsuccessful in preventing the onset of scurvy from which their animals ultimately died. As the treated lemon juice described above contains very little extraneous matter highly potent doses can be administered. It was of interest to see if by injecting concentrated doses of the treated juice subcutaneously into guinea pigs suffering from scurvy, the animals could be cured. Three guinea pigs were employed for the purpose and the following protocols describe the experiment.

Guinea pig No. 67. When put on a scorbutic diet it weighed 261 g. After subsisting on this diet for nineteen days it received a subcutaneous injection of 15 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight of the animal kept on decreasing in the usual way after the treatment and it died two days later, weighing 271 g. The post mortem examination revealed a very acute condition of scurvy.

Guinea pig No. 58 A. When put on scorbutic diet it weighed 467 g. After twenty-one days it received a subcutaneous injection of 10 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight kept on decreasing and the animal died four days after the treatment. The post mortem examination revealed an acute condition of scurvy.

Guinea pig No. 66. When put on a scorbutic diet it weighed 845 g. After twenty-five days it received a subcutaneous injection of 10 cc. of concentrated treated lemon juice equivalent to 100 cc. of the original lemon juice. The weight of the animal kept on decreasing and it died three days later. The post mortem revealed a very mild condition of scurvy.

It is seen from the above experiments that when guinea pigs are suffering from well declared scurvy subcutaneous injection of concentrated doses of an antiscorbutic does not produce any amelioration in the condition of the animal and the progress of the disease is unaltered.

Expt. 7. This experiment was instituted with the purpose of ascertaining whether by administering high doses of antiscorbutic before putting animals on a scorbutic diet any prophylaxis could be achieved. By using concentrated doses of treated lemon juice very large quantities of very high potency could

be administered within a few days which it would have been impossible to give with the corresponding original lemon juice. The following protocols describe the actual experiment.

Guinea pig No. 80 received within four days concentrated doses of treated lemon juice equivalent to 1280 cc. of original lemon juice. It was then put on a diet of oats, bran and autoclaved milk. Its weight at the commencement of the experiment was 375 g. After fourteen days it began to decrease in weight and died after it had existed twenty-four days on the scorbutic diet. At the time of its death it weighed 259 g. The post mortem examination revealed acute scurvy.

Guinea pig No. 81 received within five days concentrated doses of treated lemon juice equivalent to 1050 cc. of original lemon juice. Its weight at the time it was put on a scorbutic diet of oats, bran and autoclaved milk was 359 g. It began to decrease in weight after eighteen days and died twelve days later. At the time of its death it weighed 199 g. The post mortem examination revealed acute scurvy.

Guinea pig No. 82 received within four days concentrated doses of treated lemon juice equivalent to 900 cc. of the original lemon juice. It was then put on a scorbutic diet of oats, bran and autoclaved milk. Initial weight was 358 g. After sixteen days it commenced to decrease in weight and died eight days later. Its final weight was 238 g. The post mortem examination revealed very acute scurvy.

The administration then of high doses of antiscorbutics previous to a period of deprivation of the antiscorbutic factor does not prevent or delay the onset of scurvy in guinea pigs, as the protocols show that the animals behaved in the same way as animals kept on a scorbutic diet without receiving any previous treatment.

Expt. 8. It was shown by Hart and Lessing [1913] that scurvy can be induced in monkeys. The clinical picture and course of the disease in these animals is almost identical with that of human beings. We too have managed to induce scurvy in monkeys by using a diet which was theoretically complete in every respect but lacked the antiscorbutic element and the description of these experiments will be communicated in detail elsewhere. In view of the fact that all our experiments had been carried out on guinea pigs it was also desirable to study the curative action of the treated lemon juice on a scorbutic monkey. A *Macacus rhesus* monkey, estimated to be about two years of age, suffering from acute experimental scurvy was submitted to the treatment. The details of the experiment are given in the following protocol.

Monkey No. 6 (female *Macacus rhesus*) was put on a scorbutic diet on October 18. Incipient symptoms of scurvy observed on December 29. From that time the disease became gradually worse. On January 29 the animal was in an acute scorbutic condition showing haemorrhaged eyelids, loose teeth with disintegrating enamel. The gums very spongy and haemorrhaged. The animal was almost unable to use its legs. It then received a dose of treated lemon juice by means of the stomach pump. For the following five days it received convenient doses of the treated lemon juice which amounted altogether to the equivalent of 980 cc. of the original lemon juice. On January 24, the day following the administration of the first dose, the teeth became clearer. On January 25 the condition of the gums was markedly improved and the teeth were decidedly firmer. The improvement continued and by January 28, five days after the commencement of the treatment, the gums were normal, the teeth almost firm and the animal was using its legs with alacrity. The condition of the animal soon became normal. It was now receiving a daily dose of treated lemon juice equivalent to 10 cc. of the original juice besides the original scorbutic diet. The monkey throve well on this supplemented diet. On March 16 it was chloroformed and at the post mortem examination, with the exception of a deformed wrist which was evidently the vestige of a fracture which had taken place in the acute scorbutic condition, everything was found to be normal. The histological examination of the costochondral junctions in the ribs did not disclose any perceptible abnormality.

This experiment definitely confirms the potent antiscorbutic activity of the treated lemon juice described in this communication.

Summary.

1. By eliminating the free citric and other organic acids in lemon juice an antiscorbutically active residue is obtained.

2. Comparative experiments show that the best part if not the whole of the antiscorbutic content of the lemon juice is present in this fraction.

3. After storing the treated lemon juice for about a fortnight in the cold room a marked loss can be observed in its potency although it still remains fairly active.

4. By evaporating the treated lemon juice to dryness in acid medium an active dry residue is obtained.

5. Subcutaneous injections of very potent doses of treated lemon juice failed to arrest the progress of well declared scurvy in guinea-pigs.

6. The administration of very concentrated doses of treated lemon juice previous to depriving guinea pigs of the antiscorbutic factor did not prevent or delay the onset of scurvy.

7. The very high curative effect of the treated lemon juice on a scorbutic monkey has been demonstrated.

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Hart and Lessing (1913), *Der Skorbut der kleinen Kinder.*
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XXIII. NOTE ON THE ETIOLOGY OF SCURVY IN GUINEA-PIGS.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received July 5th, 1918.*)

As a result of experiments carried out on rats and guinea-pigs McCollum and Pitz [1917] came to the conclusion that scurvy in the guinea-pig is not a disease brought about by the deficiency of an accessory factor in the diet but that the scorbutic condition is caused by a diet chemically and physically unfavourable. It is particularly the physical condition of the scorbutic diet of oats which is supposed by them to have a detrimental effect on the digestive tract of the guinea-pig, causing constipation and thus facilitating the absorption of toxins produced by the bacterial flora of the intestine. These workers were unable to produce scurvy in rats by feeding them on diets of which oats formed the basal constituent, they were however able to induce scurvy after a certain period only in some of their guinea-pigs which were fed on rolled oats and fresh whole milk. Adopting that technique McCollum and Pitz tried a variety of substances which were intended either to obviate constipation in their experimental guinea-pigs or to attenuate the bacterial development in the alimentary tract of the animals. As some of the substances tested produced a beneficial result the authors considered their hypothesis to be substantiated by their observations. Such conclusions however are not in our opinion warranted by the experimental evidence given in their paper. Fresh whole milk was given to the animals *ad lib.* Milk if not subjected to any heating is antiscorbutic and it has been shown recently by Chick, Hume and Skelton [1918] that if large enough quantities of fresh milk are taken by guinea-pigs scurvy does not supervene. We agree with the suggestion of the last mentioned investigators that had the quantities of milk consumed by the animals in McCollum and Pitz's experiments been measured, the beneficial effect observed in their animals treated with the various substances could in all probability have been traced to the antiscorbutic potency of the augmented quantities of fresh milk consumed.

McCollum and Pitz, in the same communication, ascribe the antiscorbutic activity of orange juice to its content of sodium and potassium citrates which

possess laxative properties and to the retardation of the development of bacteria by the action of citric acid contained in the juice. These explanations appear hardly feasible in view of the observations made by us in connection with the antiscorbutic content of lemon juice. In the preceding paper [Harden and Zilva, 1918] we have shown that by removing the free citric acid from lemon juice a residue is obtained whose inorganic content is almost insignificant, which nevertheless contains almost the entire antiscorbutic content of the original juice. It is therefore evident that the activity of the extract cannot be ascribed either to its content of citric acid or of sodium and potassium citrates as the inorganic residue of the preventive dose of 1·5 c.c. only corresponds to about 2–3 mg. of potassium or sodium citrate.

Pitz [1918] in a recent communication describes experiments which in his opinion offer further evidence in support of the hypothesis that scurvy in the guinea-pig is not due to the absence or deficiency of a certain factor in the diet but to the absorption of toxic products of bacterial activity brought about by the long retention of the faeces in the caecum caused by the unfavourable physical consistency of the diet. In his experiments he adopted the procedure of feeding the guinea-pigs with different sugars in order to induce an acidophilic bacterial flora instead of the usual putrefactive one and thus to obviate the possibility of the animals absorbing the toxic products of putrefaction which in his opinion are responsible for the production of scurvy in guinea-pigs. He claims to have achieved quite a decided success by adding lactose to the animals' diet; with other carbohydrates a marked beneficial effect was also observed. In this investigation Pitz again employed the same technique as was used by him previously in collaboration with McCollum, giving the guinea-pigs rolled oats and fresh whole milk *ad lib.* as a basal diet. We are therefore again unable to interpret Pitz's results in the same way as he does. Moreover we had records of some experiments at the time of the appearance of Pitz's paper which did not bear out that author's observations. In connection with another investigation we had the opportunity of feeding guinea-pigs on cane sugar, fructose, and a syrup obtained after the fructose has been crystallised out from the products of the hydrolysis of inulin for which we are indebted to Prof. J. C. Irvine. These sugars, in the quantities we used, did not show the slightest antiscorbutic potency. Since the appearance of Pitz's publication we have also tested lactose, using high quantities and employing our technique, and as was expected no antiscorbutic activity was found to be possessed by the sample of the lactose used by us. We therefore consider it of interest to publish the record of our experiments.

Experimental.

In testing the antiscorbutic potency of a substance the following technique is employed in our laboratory. Guinea-pigs weighing from 250 g. to 450 g. are put on a basal diet of crushed oats and bran (one volume of oats to two volumes of bran) and a daily ration of 50 c.c. of milk autoclaved for one hour at 120°. On this diet the animal commences to drop in weight after about fourteen days and usually dies of scurvy within thirty days. In our experience no animal has ever yet failed to die of scurvy on such a diet. The substance to be tested is administered in daily rations *per os* or in some cases it is dissolved in milk which is quantitatively consumed by the animal. Fig. 1 represents the weight curves of guinea-pigs which received the scorbutic diet described above and

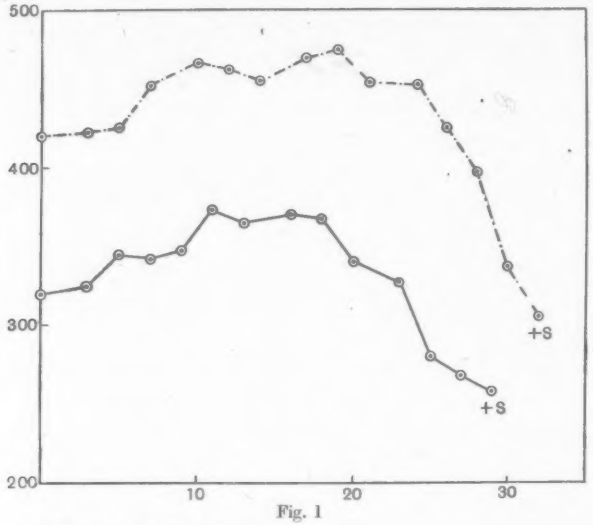

Fig. 1

⊙–.–⊙–.⊙–.–⊙ Schering's Fructose.
⊙——⊙——⊙ uncrystallisable residue from the preparation of fructose from inulin.

1 g. daily of Schering's fructose and of the uncrystallisable residues from the preparation of fructose from inulin respectively. Both these sugars were dissolved in the milk and were fully consumed by the animals until the last few days when, owing to their grave condition, the guinea-pigs were unable to consume much food. These animals died twenty-eight and thirty-four days after the commencement of the experiment and the post mortem examination disclosed a very acute condition of scurvy.

We have carried out no special experiments with cane sugar, but in the course of our work on scurvy we have on numerous occasions used generous doses of cane sugar for sweetening purposes without noticing the slightest beneficial effect, and we are convinced that it is scorbutically inactive.

Lactose was tested on three guinea-pigs receiving a basal diet of oats and bran and 50 c.c. of autoclaved milk. As in Pitz's experiments high doses were given. Two guinea-pigs received 6 g. of lactose and one 3 g. per diem in their milk. As will be seen from Table I about four-fifths of this was consumed daily. All the pigs died of scurvy within thirty days so that the onset was not even delayed. Fig. 2 shows the weight curves of the animals in question, which are typical weight curves of guinea-pigs kept on a scorbutic diet of oats, bran and autoclaved milk. A few days before their death the animals lost their inclination for food. In order to insure high consumption of lactose even at that stage the oats and bran were also mixed with lactose as well as the autoclaved milk.

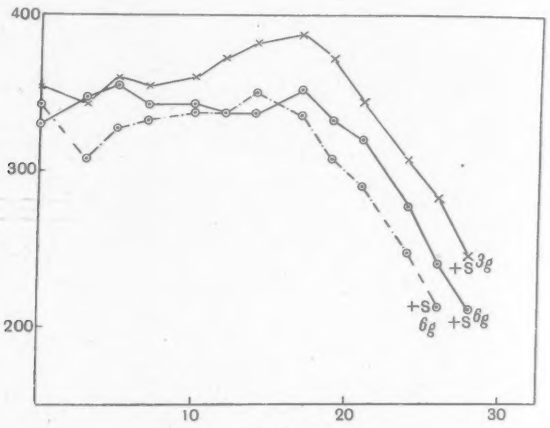

Fig. 2

It is evident from the above experiments that the sugars tested afford no protection whatsoever against scurvy, and the data tend further to strengthen our belief that any amelioration observed by McCollum and Pitz in their experimental animals was due to the enhanced consumption of the raw milk and not to the antiscorbutic potency of the substances tested. As long as these observers continue to employ their present technique we consider that their results are open to criticism.

TABLE I.

	Pig No. 269 (3 g. lactose)		Pig No. 270 (6 g. lactose)		Pig No. 271 (6 g. lactose)	
Days	Milk	Oats and bran	Milk	Oats and bran	Milk	Oats and bran
1	40 c.c.	19 g.	38 c.c.	24 g.	35 c.c.	10 g.
2	40	14	40	31	40	25
3	40	31	45	29	45	24
4	35	16	35	21	40	21
5	50	18	27	25	35	19
6	45	18	40	26	35	21
7	40	17	40	27	45	19
8	40	16	45	26	45	18
9	44	16	45	23	50	21
10	40	14	35	21	40	26
11	40	9	10	26	45	24
12	10	14	25	28	45	22
13	30	16	40	25	40	21
14	45	14	35	23	45	19
15	40	11	35	24	30	19
16	30	16	40	18	45	21
17	50	14	45	18	40	16
18	45	6	30	8	40	11
19	30	6	30	10	40	11
20	45	4	30	6	30	6
21	35	6	25	12	30	1
22	30	3	25	6	25	0
23	35	7	15	7	35	0
24	25	5	20	5	28	5
25	20	5	15	5	40	5
26	10	5	10	2	10	3
27	12	1	—	—	6	2
28	0	1	—	—	10	3
29	10	1	—	—	0	2
30	—	—	—	—	0	0

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XXIV. ON THE IMPORTANCE OF GEOLOGICAL FACTORS IN SEWAGE PURIFICATION.

By EVELYN ASHLEY COOPER and ALBERT EDWIN COOPER.

(*Received July 10th, 1918.*)

A great deal of work both from the scientific and the practical points of view has been carried out in connection with the biochemistry of sewage-purification by Letts and Adeney and other investigators for the Royal Commission on Sewage Disposal [1908, p. 5].

It is unnecessary to summarise the results of their work fully, but it may be briefly said that the investigations were concerned first, with studying the nature and course of the complex biochemical processes that go on when organic matter is decomposed by bacterial action, and the influence of various physical conditions thereon, and second, with elaborating and standardising the dissolved oxygen absorption test, commonly called the Rideal-Stewart test, in order to make this test of value in forming an opinion of the quality of a sewage effluent.

The majority of biochemists are now convinced that, although this test takes five days to complete when properly carried out, yet it yields most valuable results; and, as the bacterial processes involved are natural processes going on in river and other waters, it is to be preferred to the various other determinations often used in the standardisation of effluents, *e.g.* albuminoid ammonia, organic nitrogen, oxygen absorbed from permanganate, which are purely arbitrary and artificial and have no relation to the biological aspect of the question. The dissolved oxygen absorption test consists in measuring the amount of oxygen used up in a given period in the bacterial oxidation of the organic matter contained in the sewage effluent. According to Adeney this measures the amount of readily oxidisable carbonaceous matter present in the effluent, very little or no ammonia being oxidised during the five days' period usually given. As it is the quickly fermentable organic matter that will seriously pollute and deoxygenate a river-water, the value of this test is at once evident.

In standardising the dissolved oxygen absorption test the influence of various factors, *e.g.*, time, temperature, dilution, was investigated by the Commissioners [1913, p. 93], and it was recommended that the test should be carried

out for five days at 18° C. The period of five days was chosen, because it appeared that sometimes there was an initial inhibition of the bacterial processes, so that low results might be obtained if shorter periods were employed, and the test would then be too lenient. The standard temperature of 18° C. was selected because it was found to be unusual for river waters in this country to exceed it.

The dilution chosen was 1 in 5, as this was a convenient dilution, and it was found that up to 1 in 5, dilution had little effect on the rate of absorption of dissolved oxygen. The method of analysis is fully given.

Although this test has been worked out in some detail the question as to whether the rate of biological oxidation of the organic matter is affected by the geological source and mineralogical constituents of the water used for dilution of the effluent has not apparently been sufficiently studied. The Commissioners [1913, p. 93] recommend that good tap-water should be employed, but it is obviously difficult to define this expression. In an addendum to another report [1908, p. 433] however it is pointed out that the results may depend on the nature of the diluting water, but examples are not given, except in the case of sea-water, which, although it has no measurable effect on the carbonaceous oxidation and production of nitrite from ammonia, yet was found to prevent nitrification most effectively. Fowler [1912] also states that in the case of a sewage effluent discharged into a river-water, nitrification was prevented by calcium chloride that was discharged into the same river from an ammonia-soda factory.

The importance of geological factors was suggested by some accidental observations at this laboratory. There being two separate water-supplies laid on, the question arose as to which should be employed for dilution of the effluents in carrying out the dissolved oxygen absorption test. One water was hard and calcareous, pumped up from the upper chalk, covered in the particular district by a considerable thickness of tertiary strata (Reading beds, London Clay and Bagshot Sands). The other water was a soft ferruginous upland surface water from the gravels and underlying upper Bagshot Sands, being thrown out by the clayey Bracklesham beds underneath.

The influence of various waters on the rate of dissolved oxygen absorption by sewage effluents.

In the experiments detailed below effluents were diluted with different waters, distilled water, tap waters, and stream waters. The samples of the latter were always taken from the streams into which the effluents were being

discharged. For each set of experiments the conditions were kept constant throughout: dilution, temperature, volume, and period of incubation.

The experimental method consisted in incubating the aerated dilutions of the effluents in well-stoppered bottles for five days at 18° C. and estimating the concentration of dissolved oxygen left at the end of this period. The initial concentration of oxygen was determined by a separate test, and by difference the amount of oxygen absorbed or used up in the oxidation of the organic matter could be calculated.

A large number of experiments on these lines have been carried out over a period of a year. In order to economise space, however, only a few typical results are tabulated in this paper.

The main points emerging from the work are as follows.

I. Practical.

The Standardisation of Sewage Effluents.

1. The dissolved oxygen absorption figure (five days) for a sewage effluent diluted with hard tap-water (derived from the chalk) was only from one-twentieth to one-half the figure obtained for the same effluent diluted with distilled water, or stream water taken from the stream into which the effluent was discharged.

The following results illustrate this:

Dissolved oxygen absorbed by 100,000 parts of effluent. Dilution made with		
(1) Distilled water	(2) Hard water	(3) Stream water
2·15	0·45	—
1·25	0·17	0·45
8·1	0·46	4·2

2. The dissolved oxygen absorption figure (five days) for an effluent may be considerably lower when the effluent is diluted with stream water than when diluted with distilled water.

Dissolved oxygen absorbed by 100,000 parts of effluent. Dilution made with	
(1) Distilled water	(2) Stream water
1·25	0·45
2·36	1·20
8·1	4·2

In examples 1 and 2 the stream water was taken from a stream flowing over the Hythe and the Folkestone beds, and in example 3, from a river in the Bagshot Sands, receiving hard surface drainage water and sewage from a town.

3. The dissolved oxygen absorption figure (five days) of an effluent was somewhat lower, about 20 %, when dilution was made with a ferruginous peaty tap-water (upland surface supply) from gravel and upper Bagshot Sands than when made with distilled water.

4. The biological oxidation of sewage was almost entirely stopped for forty-eight hours by dilution with a very ferruginous water from the Folkestone beds (iron content of water: 2·5 parts per 100,000). No inhibition, however, at all was noticed after five days. There was no initial inhibition when the iron had been previously removed from the water by means of a Candy-filter.

The following results illustrate this:

	Dissolved oxygen absorbed by 100,000 parts of effluent.	
	48 hours	5 days
Dilution with distilled water	0·92	2·16
„ „ ferruginous water	0·08	2·10
„ „ filtered ferruginous water	0·92	2·06

The addition of soil taken from the ferruginous strata (iron content: 10 %) near the junction of the Hythe and Folkestone beds also inhibited the biological oxidation of sewage.

It is evident therefore that the results obtained with waters of different geological source are most divergent, and it is essential for the routine examination of sewage effluents that a fixed standard be adopted; otherwise the results may vary considerably according to the laboratory in which the effluents are examined and the tap-water employed. At first it might be thought that distilled water should always be used as a diluting medium in the standardisation of effluents, because it is independent of geological conditions and the only factors involved are the organic matter and mineralogical constituents of the sewage itself. The chief purpose however of standardising a sewage effluent is to ascertain whether the effluent is likely to exert a serious polluting effect on the stream or river into which it is being discharged. It is therefore necessary that the experimental conditions should approximate as closely as possible to the natural conditions. It seems reasonable therefore to use the river or stream water as the diluting medium, the sample of water being of course collected well above the point at which the effluent is discharged. It may be expected that different results will be obtained by using different river waters, but if for each effluent to be examined the diluting water is always taken from the river or stream into which the particular effluent enters, consistent results will be obtained, and the test will be carried out under

well standardised conditions. If the river water is taken as the standard diluting medium, the use of hard tap-water or a strongly ferruginous water will clearly make the oxygen test far too lenient. On the other hand, the use of distilled water may render the test somewhat severe, and thus cause effluents to be condemned that would not in reality seriously pollute the water-way.

It is further suggested that in the routine examination of sewage effluents more attention should be given to the conditions of the river or stream below the point of discharge. As a check on the effluent test, it is desirable to determine regularly the dissolved oxygen absorption figure for the undiluted water collected from the river or stream well below the point of pollution, care being taken to ensure that the sample is taken where the effluent and river water are thoroughly mixed.

According to the report of the Sewage Commission [1913, p. 132] the dissolved oxygen absorption figures of waters unpolluted by sewage were always well below 0·4 part of oxygen per 100,000, and, if this limit were exceeded owing to the sewage pollution, conditions were likely to become offensive. It was recommended therefore that sewage purification should be sufficient to prevent an effluent from being of so bad a quality, that the Rideal-Stewart figure of the stream water below the point of pollution exceeded 0·4 part oxygen per 100,000. For average dilution this would mean that the Rideal-Stewart figure for an effluent should not exceed 2·0. This standard however is adaptable to conditions; if the stream were very small, a more stringent standard would have to be adopted; while in the case of a large river, considerable latitude could be given. During the past year many laboratory and field observations have been made in the case of numerous streams in Surrey and Hampshire, chiefly tributaries of the river Wey. As the result of these observations it has been possible to confirm the conclusion that for unpolluted waters the dissolved oxygen absorption figure does not exceed 0·4, and that where effluents are discharged into water-ways, in order to avoid nuisance, the effluents should be of such quality that the streams are kept well within the above limit.

2. Theoretical.

So far only the practical question of standardisation of effluents has been considered. We have also to enquire into the cause of the marked inhibitory effects described in the previous section. As already stated the hard tap-waters that were found to inhibit the biological oxidation of sewage were municipal water supplies obtained from borings in the chalk, which was covered in the particular districts by the Woolwich and Reading beds, London Clay and

Bagshot Sands. The waters contained a considerable amount of calcium bicarbonate and calcium sulphate (total hardness 24·0, permanent hardness 6·5, temporary hardness 17·5). Magnesium however was found to be absent. When the hard water was deprived of all calcium carbonate by boiling and filtering, it no longer exerted an inhibitory action on the processes of biological oxidation. This was taken as presumptive evidence that the inhibitory factor was calcium bicarbonate. If this were the case, it was thought that the addition of solid calcium carbonate should also inhibit the oxidation of a sewage effluent, as there should be sufficient carbon dioxide present to form soluble calcium bicarbonate. Preliminary experiments however showed that the biological oxidation was apparently accelerated by the addition of calcium carbonate. This was not due to impurities in the latter, as by itself it did not use up any dissolved oxygen. It was noticed however that when chalk was added to a sewage effluent, the initial titrations came out about 40 % lower, *i.e.*, there was an apparent loss of dissolved oxygen in the short time (thirty minutes) occupied in the estimations. It was ascertained by syphoning the liquid free from chalk before titration, that various chalks, *e.g.*, pure calcium carbonate, chalk from Upper, Middle and Lower Chalk Formations, and also chalk marls, did not adsorb dissolved oxygen from aqueous solution, and that accurate results were always obtained if care were taken to remove the chalk by syphoning off the supernatant liquid before estimating the concentrations of dissolved oxygen. It would appear that when the chalk was not removed before the titrations some of the dissolved oxygen was carried out of solution mechanically during the effervescence due to the CO_2 liberated by the action of the sulphuric acid added upon the chalk.

Subsequent experiments in which the precaution of removing the chalk before the commencement of the estimation of the dissolved oxygen was always taken showed that the addition of calcium carbonate had practically no effect on the rate of biological oxidation of sewage. The addition of magnesium carbonate had however a very pronounced inhibitory effect. There being no magnesium present in the hard waters used in the experiments described above, nothing definite can thus be said at present as to why these hard waters when added to sewage so strongly inhibited the processes of biological oxidation; and further work must be carried out on this subject.

In the case of ferruginous water from the Folkestone beds which was found to inhibit temporarily the oxidation processes, it is probable that the inhibitory factor was ferrous carbonate, which is known to be present in such waters, and to have a toxic effect on organisms.

Main Conclusions.

The work so far described is only of a preliminary nature, but is sufficiently advanced to show that in the dissolved oxygen absorption test for the standardisation of a sewage effluent, in order to obtain reliable results, it is important to employ, as a medium for diluting the sewage effluent, water collected from the river or stream into which the effluent is discharged, instead of the laboratory tap-water which is commonly used.

It is further recommended that, as a check in routine work, the river or stream water below the point of pollution be regularly tested, in order to ascertain if its dissolved oxygen absorption figure be below or above the limit of 0·4 part per 100,000.

It is too early yet to define the precise conditions and the extent to which geological factors may influence the processes of sewage purification. The results given in this paper have been obtained in connection with experimental work on sewage effluents in a restricted area, where the tap-water was yielded by sedimentary rocks of a simple kind—chalk, gravel, and sands. These results however suggest that, under different geological conditions, waters from other sedimentary rocks, and also waters associated with the detritus of eruptive rocks, where the active chemical constituents have been or are released may be expected to affect profoundly the biological processes of oxidation.

The practical biochemical work of this investigation has been carried out by one of us (E. A. C.) at the Hygienic Laboratory, School of Army Sanitation, Aldershot, while the geological aspect of the subject has been worked out by A. E. C.

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